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Guidelines

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Authors
Jan Pawlowski[1,2], Laure Apothéloz-Perret-Gentil[1,2], Elvira Mächler[3,4], Florian Altermatt[3,4]

[1] University of Geneva, Department of Genetics and Evolution, 1211 Geneva
[2] ID-Gene Ecodiagnostics, 1202 Geneva
[3] Eawag: Swiss Federal Institute of Aquatic Science and Technology, Department of Aquatic Ecology, 8600 Dübendorf
[4] University of Zurich, Department of Evolutionary Biology and Environmental Studies, 8057 Zürich

Contact
Jan.Pawlowski@unige.ch and Florian.Altermatt@eawag.ch

Advisory group
Marie-Sophie Renevier, Yael Schindler Wildhaber, Arielle Cordonier, Lukas De Ventura, Daniel Hefti, Christiane Illg, Glenn Litsios, Philip Staufer, Patrick Steinmann

External reviewers
Rosetta C. Blackman, Kristy L. Deiner, Florian Leese, Kristian Meissner

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Table of contents

Abstracts

Aquatic biomonitoring is currently transformed by environmental DNA (eDNA) based approaches. These new tools overcome some limitations of traditional biomonitoring and allow non-invasive sampling, broad taxonomic coverage, high sensitivity, and the possibility to automation. However, the disruptive character and rapid developments of the new technology challenge its implementation. This publication explains the principles of the eDNA technology and presents its advantages and limitations. It shows possible applications of eDNA tools in monitoring and assessment of aquatic ecosystems, and provides detailed protocols and best practices for processing eDNA samples.

Keywords:
Biodiversity, environmental indicators, monitoring, method guidelines, eDNA, method standardization.

Das Biomonitoring aquatischer Lebensräume wird derzeit durch Verfahren, die auf Umwelt-DNA (eDNA) basieren, verändert. Diese neuen Instrumente überwinden gewisse Beschränkungen herkömmlicher Biomonitoringmethoden und erlauben eine nichtinvasive Probenahme, eine breite taxonomische Auflösung, eine hohe Sensitivität und die Möglichkeit, Prozesse zu automatisieren. Allerdings stellen die komplett neue Herangehensweise und die rasche Entwicklung der neuen Technologie Herausforderungen für ihre Einführung in die Praxis dar. In dieser Publikation werden die Grundsätze der eDNA-Technologie erläutert und die Vorteile und Beschränkungen vorgestellt. Es werden mögliche Anwendungen von eDNA-Tools für das Monitoring und die Bewertung aquatischer Ökosysteme aufgezeigt und detaillierte Protokolle und bewährte Praktiken für die Verarbeitung von eDNA-Proben vorgestellt.

Stichwörter:
Biodiversität, Umweltindikatoren, Monitoring, Methodenrichtlinien, eDNA, Methodenstandardisierung

Les approches fondées sur l’ADN environnemental (ADNe) sont en passe de transformer la biosurveillance aquatique. Ces nouveaux outils permettent d’outrepasser les limites de la surveillance biologique traditionnelle : ils permettent d’effectuer un échantillonnage non invasif, de couvrir un large éventail taxonomique et offrent une sensibilité élevée ainsi que des possibilités d’automatisation. Cependant, le caractère révolutionnaire et les développements rapides de cette nouvelle technologie entravent sa mise en œuvre. La présente publication explique les principes des méthodes ADNe, en présente les avantages et les limites et formule des suggestions concernant les standards et les pratiques de routine. En outre, elle montre les utilisations possibles des outils fondés sur l’ADNe dans la surveillance et l’évaluation des écosystèmes aquatiques, expose des études de cas spécifiques et propose des protocoles détaillés ainsi que des exemples de bonnes pratiques pour le traitement des échantillons d’ADNe.

Mots-clés :
biodiversité, indicateurs environnementaux, surveillance, directives méthodologiques, ADNe, standardisation des méthodes

Il biomonitoraggio acquatico sta passando ad approcci basati sul DNA ambientale (eDNA). Questi nuovi strumenti superano determinati limiti del biomonitoraggio tradizionale e consentono un campionamento non invasivo, un’ampia copertura tassonomica, sensibilità elevate e la possibilità di automazione. Tuttavia, il carattere dirompente e il rapido sviluppo delle nuove tecnologie mette a dura prova la sua attuazione. La presente pubblicazione spiega i principi della tecnica eDNA en ne presenta vantaggi e limiti. Inoltre, illustra possibili applicazioni degli strumenti eDNA nel monitoraggio e nella valutazione di ecosistemi acquatici, fornisce protocolli dettagliati e buone pratiche per il trattamento di campioni di eDNA.

Parole chiave:
Parole chiave: biodiversità, indicatori ambientali, monitoraggio, linee guida metodologiche, eDNA, standardizzazione dei metodi.

Foreword

In Switzerland, aquatic ecosystems and the species living in them are currently under great anthropogenic stress. Accordingly, the highest proportions of extinct or endangered species are found among aquatic organisms. The aim of the Waters Protection Act (WPA, SR 814.20) of 24 January 1991 is to protect waters against harmful effects. Moreover, the Waters Protection Ordinance of 28 October 1998 sets out the ecological goals for waters (Annex 1 WPO, SR 814.201). Assessing the biological quality of watercourses is crucial for meeting these ecological goals.

To analyze and evaluate the aquatic biocenosis, a dataset of sufficient quantity and quality is required. However, acquiring such data is not always possible with currently available methods. Environmental DNA (eDNA) techniques can help to address this problem. A lot of important information about the biological status of aquatic ecosystems can be obtained from simple water or sediment samples. eDNA techniques have many advantages, interest in them is growing and many methods are being developed around the world. This rapid development implies that it is currently difficult for practitioners and decision makers to know what methods are available, which ones can be used for aquatic biomonitoring and where information about systematic approaches can be found.

These guidelines on 'Environmental DNA applications in biomonitoring and bioassessment of aquatic ecosystems' are intended to promote and support the standardization and implementation of eDNA methods in aquatic biomonitoring and in assessing the status of aquatic ecosystems. They are aimed at practitioners and decision-makers (government, cantons, engineering firms). To this end, the document provides an overview of the various methods available, discusses the advantages and disadvantages of eDNA methods and gives suggestions for recommended best practices and routine standards.

The FOEN would like to thank everyone involved in the publication of this guide, in particular the authors, the expert group and the cantonal representatives, all of whom made an invaluable contribution.

Stephan Müller
Head of Water Division
Federal Office for the Environment (FOEN)

1 Introduction

Well-functioning and intact aquatic ecosystems are essential for human well-being, providers of a variety of ecosystem services, and contain an exceptional diversity of organismal life. However, human activities, such as pollution, intensification in land-use, invasive species, or use of water for energy production are threatening the state and functioning of freshwater ecosystems at a local to global scale (Benateau et al., 2019; Reid et al., 2019). The understanding, management and protection of aquatic ecosystem is thus of highest priority. In Switzerland, the federal law from January 24th 1991 on the protection of waters (*Gewässerschutzgesetz,* GSchG, SR814.20) asks in article 57 and 58 the federal government and cantons respectively to clarify the state of water bodies. This specifically includes evaluations if the ecological states and their goals for aquatic ecosystems are reached as specified in the *Gewässerschutzverordnung* (water protection regulation, GSchV, SR 814.201). Annex 1 cipher 1 paragraph 1 GSchV states that ecological communities of plants, animals and microbes of surface water bodies are near-natural, self-regulated and represent a diversity and abundance of species typical for pristine or low-affected water bodies of that type. This allows the protection of water bodies from negative influences, such that a sustainable use and good ecological status can be maintained. The federal government and cantons conduct monitoring to ensure that the requirements on water quality of surface waters in annex 2 cipher 1 GSchV are fulfilled. Central to this are good data on the state but also change of aquatic ecosystems, and respective variables describing individual components of these systems.

An adequate monitoring of aquatic ecosystems is thereby essential, and has a long tradition, both with respect to variables looked at and methods used. Aquatic ecosystems, ranging from ponds and lakes to streams and rivers, can be assessed based on abiotic aspects, including water chemistry and physical structure, or based on biotic aspects, including the diversity and composition of biological communities that are representative for focal endpoints. Importantly, any of these monitoring approaches assume that measuring a few key variables will describe the state, and possible direction of change, of the whole ecosystem. Thus, endpoints are proxies and simplified descriptors of a more complex system.

The development and use of monitoring approaches for endpoints has a long history, and has gradually grown over the last decades. While simple chemical assessments on macronutrients dominated early on, they were complemented by biological endpoints characterizing the nutritional loading of freshwater systems (e. g., Saprobic index), and subsequently by parameters describing structural modifications and pollution by diverse chemicals, and a series of taxonomic groups, such as fish, macroinvertebrates, or diatoms, capturing these effects. Importantly, the use of monitoring approaches, and the specific tools needed, have grown organically over the last decades, both driven by specific needs requiring new endpoints (e. g., new drivers, such as micropollutants) and the techniques available. This resulted, in Switzerland and elsewhere, in a set of commonly applied standards (e. g., BAFU 2019a), well-reflected in large monitoring programs, such as the *Nationale Beobachtung Oberflächengewässerqualität NAWA* (BAFU, 2013; Kunz et al., 2016) or the *Swiss Biodiversity Monitoring* (BDM) program (BDM Coordination office, 2014). Many of these programs are operational and well-functional (Wüthrich & Altermatt, 2019). However, they also all have inherent limitations and challenges, mostly caused by the methods used. For example, most of them rely on the sampling, sorting and morphological identification of organisms, which is time-consuming and can only be done for a small set of organismal groups. Also, many of the techniques can only be applied to wadeable waterbodies, and are not applicable to large rivers and lakes, and are also hard to apply to very small streams, groundwater or spring systems. None of the currently applied techniques has the potential to be automated (neither in the sampling nor in the processing), which inherently limits analysis at finely-resolved spatial or temporal scales. From the field of aquatic chemistry, however, it has been shown that such a high temporal resolution of sampling is needed to adequately describe and understand river and stream ecosystems.

Within the last 4 – 8 years, a new player has emerged, with the potential to overcome some of these limitations and to revolutionize biomonitoring and bioassessment of aquatic ecosystems: the so-called environmental DNA (eDNA). It has been recognized that DNA from all organisms, not only microbes, can be sampled and extracted from environmental samples. This DNA, found in environmental samples including soil, water, sediment, and air, is called eDNA. The use and potential of the eDNA approach is a rapidly growing field, with techniques advancing at a high speed, and nowadays allowing sequencing of DNA at a resolution and cost completely unimagined only a decade ago. Environmental DNA-based monitoring offers several advantages. It is non-invasive for macro-organisms (specimens do not need to be sampled themselves), taxon-independent (all organisms, from bacteria to plants and animals can be potentially sampled) and has the potential to be automated (sampling and processing, allowing a high spatial and temporal resolution).

The development and use of eDNA has gained interest and applications especially in aquatic ecosystems. We are currently in a time-period in which some of these approaches are already well-established and integrated into legally binding biomonitoring schemes (e.g., on invasive Carp species in the US (US Fish and Wildlife Service 2019), and endangered newts in the UK (*www.gov.uk/guidance/great-crested-newts-surveys-and-mitigation-for-development-projects*)). At the same time the advances in biotechnologies are pushing the frontiers of what is technically possible at a pace that outdates itself on yearly intervals. In parallel, the legal and practical implementation is discussed, tested and standardized, both regionally (e.g., with several pilot projects on eDNA-use in Switzerland at cantonal and national level) and internationally (e.g., with standards being discussed in a Europe-wide COST Action *DNAqua Net* and a respective working group within the European Committee for Standardization (Leese et al., 2018)). This rapid development has not only led to the situation that the planning and potential of the technique happens "on the go", but also that hopes, expectations and promises on the ability of the technique vary widely: by some, eDNA approaches are seen as the solution to all biomonitoring problems, while by others, the focus is more on possible limitations and still ongoing method development. Thus, we have a situation where the field is in rapid development, some aspects and approaches are partially already implemented, while others are more ideas and visions, but with high potential for application in the future.

For stakeholders and practitioners, this situation is challenging as they need to decide now on what technique to use, what techniques to invest in, how to (re)direct monitoring schemes, and which promises to follow. Also, it is important to decide and agree on common norms, in order to ensure a replicable and trustable implementation. To do so in an adequate and informed manner, an outline on the different technical opportunities, both as a state-of-the-art overview as well as at the routine level, is needed. While there is an exponentially growing literature on eDNA (e.g., Rees et al., 2014a; Thomsen & Willerslev, 2015; Deiner et al., 2017), many studies are not directly applicable or accessible to stakeholders and practitioners. Next to the purely scientific literature, a series of national reports and guidelines covering aspects of new molecular practices for biomonitoring have been published. However, they are either taxon or habitat specific (e.g., Laramie et al., 2015; Carim et al., 2016; Holderegger et al., 2019) or are giving more of an overview rather than details on the specific implementation and technical aspects (Herder et al., 2014; Winding et al., 2019). A more detailed synthesis and guidelines targeting practitioners may help to set standards in the field, creating consistency between studies but also defining quality levels to be reached. Finally, it may help to give practical suggestions on how to move forward in decision making and implementation of aquatic biomonitoring.

In this report we aim to give an overview of the eDNA techniques available for (bio)monitoring of organisms in freshwater ecosystems, and to specifically discuss the pros and cons of the different techniques. Furthermore, the report gives normative suggestions on best practices and routine standards recommended. These recommendations are the current state of available knowledge, and further improvements/changes can be expected. The focus is on eukaryotes, such as fish, amphibians, macroinvertebrates, or diatoms, even though many of the statements will also be valid for bacteria. The recommendations of best practices are given at a relatively high and generalizable level. We complement these high-level recommendations with more specific protocols that reflect generally accepted and applied practices in the field to date and which can be seen as normative in a rapidly developing field. The goal of the report is to help to standardise and implement the use environmental DNA applications in biomonitoring and bioassessment of aquatic ecosystems, and ultimately contribute to their sustainable use, management and protection.

2 Environmental DNA: definitions, applications and perspectives

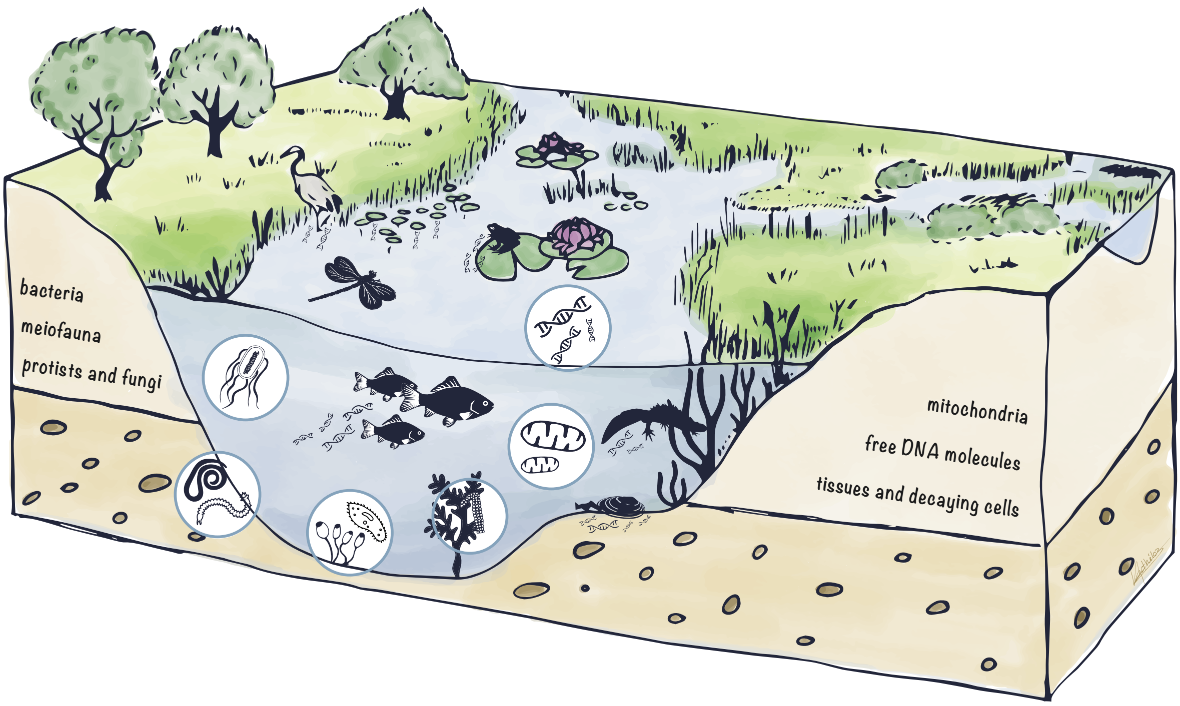
2.1 Definitions

What is environmental DNA?
Environmental DNA (eDNA) is a pool of genomic material originating from living organisms and their remains present in different types of environmental samples (water, sediment, soil, air, see Fig. 1). The major part of DNA found in environmental samples originates from single-cell microorganisms (viruses, bacteria, protists), which are generally very abundant. However, eDNA samples also comprise genomic material of multicellular organisms, either from whole small-sized organisms (zooplankton, meiofauna) or from the traces and remains of larger-sized organisms (vertebrates, invertebrates, or plants). These genetic traces of animals and plants, sometimes called *extra-organismal* or *macrobial* DNA (Barnes and Turner 2016), include reproductive stages such as gametes, tissue fragments, epithelial cells, or excretions produced or expelled by the organisms during their life cycle. They are preserved in the environment for a certain time, ranging from hours to days in the water column (Sansom & Sassoubre, 2017), to decades and centuries in sediments (Monchamp et al., 2018), and millennia in ice (Pedersen et al., 2015) and sea floor cores (Lejzerowicz et al., 2015). Collecting and analysing this eDNA allows the detection and monitoring of macrobial species, even if the organisms themselves are not actually present in environmental samples.

This report adopts a definition of eDNA *sensu lato*, which comprises DNA of different origins, including microbial and macrobial species. This is specifically done so because some routine biomonitoring programs use also single-celled bioindicators, such as diatoms. The report also considers bulk DNA extracted from samples of macroinvertebrates, obtained by kick-net or sieving.

Figure 1

All organisms potentially contribute to environmental DNA (eDNA), and eDNA can be from different origin, such as whole cells or tissue fragments, organelles or free DNA molecules. eDNA can be sampled from water, soil, sediment, or air.



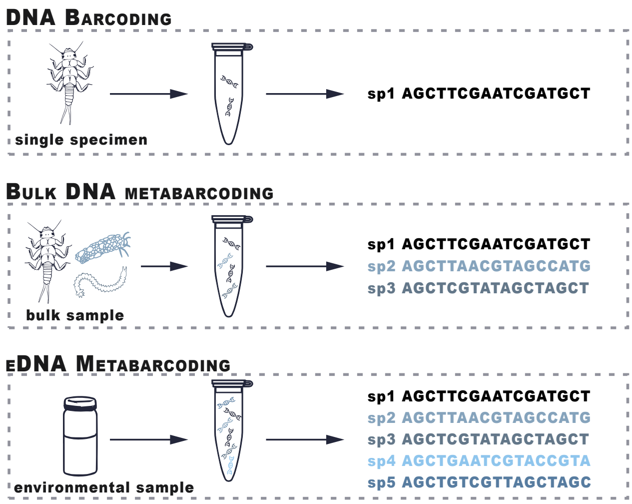
For clarity, the specific origin of DNA is clearly defined throughout the report.

What are DNA barcoding and metabarcoding?
DNA molecules contain genetic information specific to each species. Selected short fragments of DNA, called **DNA barcodes**, can be used to identify species or higher taxa depending on their level of variability. Such fragments are commonly composed of a hypervariable region and allow the use of the same barcode region for multiple species within a taxonomic group. Ideally, the DNA barcode should be variable enough to distinguish closely related species (i.e., variable at the interspecific level) but be relatively conserved within a species (i.e., conserved at the intraspecific level). There are well recognized standard barcoding genes commonly used for identification of animals (Hebert et al., 2003), plants (Hollingsworth, 2011), fungi (Schoch et al., 2012), or protists (Pawlowski et al., 2012).

Typically, each barcode is associated with a voucher specimen from which it has been obtained. The worldwide database of DNA barcodes (*www.boldsystems.org*) is managed by the International Barcode of Life (*https://ibol.org*). In Switzerland, the DNA barcodes of the Swiss fauna and flora are managed by SwissBOL (*www.swissbol.ch*) (see chapter 6).

DNA metabarcoding differs from DNA barcoding by analysing a community of species rather than a single species (Fig. 2). The community sample can be obtained from environmental or bulk samples, the latter are defined as a mixture of whole organisms originating from the environment. The number of different metabarcodes found in a sample can be very high and depends on the specificity of the barcoding gene and the diversity of species present in the environment. The main challenge of a typical metabarcoding study is to assign metabarcodes to the species or higher taxonomic categories. The efficiency of the taxonomic assignment depends on the completeness of the barcoding reference database. Gaps in barcoding reference databases are the most severe limitation in the ecological interpretation of metabarcoding data (Weigand et al., 2019).

Figure 2
Schematic explanation of barcoding, bulk and eDNA metabarcoding
In barcoding, DNA is extracted from a single specimen and a specific DNA barcode region is sequenced. In bulk DNA metabarcoding, DNA is extracted from the tissues of many specimens potentially belonging to many species. In eDNA metabarcoding, DNA is extracted directly from the environmental sample (water, soil, sediment, or air). In the two latter approaches, sequences of many different taxa will be generated, which need to be bioinformatically separated.



2.2 Potential applications

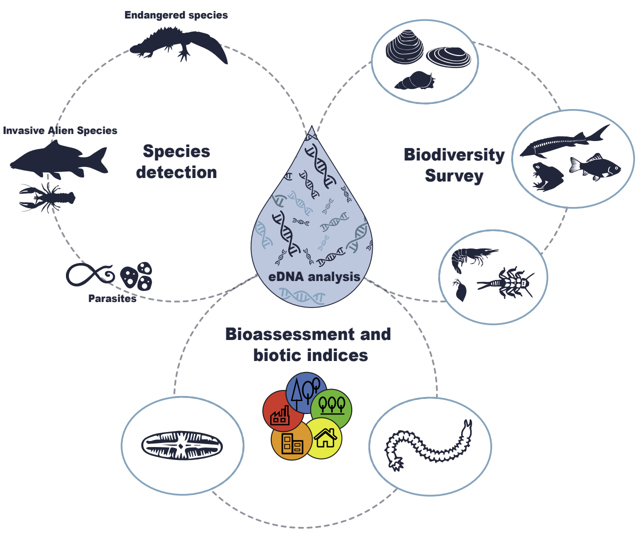
There are three major ways eDNA can be used in aquatic biomonitoring (Fig. 3):

- Single-species detection
- Biodiversity survey (community composition)
- Bioassessment (biotic indices)

Single-species detection is commonly used in conservation biology (monitoring of rare/endangered species) and the management and monitoring of biological invasions (Harper et al., 2017; Holderegger et al., 2019), or for the detection of parasites and pathogens (Krieg et al., 2019b). It might require the development of species-specific probes that allow detecting the target species. A specific advantage of the single-species method is that the amount of DNA can be quantified relatively accurately using qPCR and dPCR. The approach has been shown to be very efficient by numerous studies of invasive and endangered species of fish, amphibians, and molluscs, or studies on pathogens and parasites, detecting their DNA traces in water and sediment (Jerde et al., 2011; Mächler et al., 2014; Bass et al., 2015). Its application to crustaceans (especially crayfish, see Krieg et al. (2019a)) and external skeleton-bearing taxa such as beetles seems to be more challenging, likely due to reduced shedding of DNA into the environment and thus their eDNA falling below detection limits of these methods. The characteristics of using eDNA for single-species detection of aquatic species have been reviewed by Goldberg et al. (2016), and many others.

Figure 3

Potential eDNA applications include detection of single species, biodiversity surveys and bioassessment based on DNA extracted from environmental samples



Biodiversity survey is another common application of eDNA. In this case, the eDNA metabarcoding method is used to provide information about the composition, structure, and diversity of a community of organisms. This method is powered by high-throughput sequencing technologies that generate millions of DNA sequences and potentially allow identification of all species present in a sample, including rare and inconspicuous ones. The metabarcoding approach has been shown to provide species lists as complete as traditional methods based on electrofishing (Hänfling et al., 2016) or kick-net sampling (Fernández et al., 2018; Mächler et al., 2019). Most of eDNA-based biodiversity surveys of aquatic ecosystems are using water or sediment samples. Yet, in the case of aquatic insects and macrozoobenthos, analysing DNA from bulk samples has been proposed to be a more straightforward solution for a short-term implementation (Blackman et al., 2019). However, while it may give estimates more comparable to existing techniques than eDNA-based approaches, it propagates limitations of existing methods, such as time-consuming sampling or size-biased sampling.

Metabarcoding data can also be used for inferring **biotic indices** for environmental impact assessment (reviewed in Pawlowski et al., 2018). There are about 300 assessment methods recognized in Europe (Birk et al., 2012), among them, four are currently used in Switzerland (fish, Schager & Peter, 2004; aquatic invertebrates, Stucki, 2010; plants, Känel et al., 2017; diatoms, Hürlimann & Niederhauser, 2007). Substantial efforts have been made to calculate these indices based on eDNA data, particularly in the case of diatoms (see chapter 8.4.1). Main challenges are the incompleteness of DNA barcode reference databases (Weigand et al., 2019) and the interpretation of quantitative eDNA data. The solutions proposed to overcome these limitations are promising and some molecular indices are under development (Apothéloz-Perret-Gentil et al., 2017).

2.3 Advantages and disadvantages

The use of eDNA-based approaches has numerous advantages compared to traditional methods that are based on direct sampling of organisms and morphological identification (Table 1). Among others, eDNA allows non-invasive sampling, identification of inconspicuous and fragmented specimens, or broadening the range of indicator taxa. However, the method also has some important drawbacks that should be taken in consideration. Given that the method is rapidly developing, some drawbacks will be resolved, while others may be more inherent. For example, eDNA-based approaches may be less suitable to estimate abundances and cannot provide information on the age or size structure of a population. Furthermore, eDNA approaches do not allow to identify hybrids or recently diverged species (e.g., white-fish species of the genus *Coregonus*) that can only be identified by multi-locus genotypes or by strong linkage disequilibrium. In these latter cases, the information on the species identity is physically disconnected on multiple chromosomes, and only tissue samples from single individuals are diagnostic. This report outlines and describes the advantages and the best technological uses as of today, but also highlights where and how caution in interpretation and comparison to traditional assessments is needed.

Table 1

The pros and cons of eDNA analysis compared to traditional approaches

Many of those are especially relevant for organisms covered by the Protection of Animals Act (e.g., fishes, decapods, and amphibians).

	eDNA	Traditional sampling/Morphological identification
Time per sample	Faster for large number of samples	Fixed (i.e., little temporal optimisation possible).
Costs per sample	Decreases with more samples (metabarcoding only)	Fixed
Sensitivity	Often very high, detecting species traces, juveniles and reproductive stages	Generally low, requires large sampling efforts to obtain complete species list
Taxonomic range	Generally broad, can be applied to many taxonomic groups	Limited to taxa that can be distinguished morphologically
Detectability	Very high, useful for detection of rare, invasive and pathogenic species	Require intensive sampling
Sampling	Non-invasive, except for the bulk samples	Usually invasive (electrofishing, kick-net)
Field observations	Require using special field equipment (e.g., portable PCR)	Possible in case of large-size animals and plants
Sample processing	Complex, could be automated	Usually simple manipulations, but manual (no automation possible)
Contamination	Highly sensitive, and thus potential risk	Low risk
Infrastructure	Requires specialized molecular lab	Can be done using simple equipment
Species identification	Based on reference database, usually public Can detect cryptic species and genetic varieties	Based on personal taxonomic expertise and available literature
Qualitative data	List of species / clustered sequences (OTUs) including living organisms and their remnants	List of living species, population structure and health status
Quantitative data	Relative abundance of reads (metabarcoding) or DNA quantification (qPCR)	Absolute abundance of specimens in the sample
Data analysis	Require special bioinformatic pipelines for sequence analysis	Using relatively simple statistical tools
Data interpretation	Need to take in consideration various technical biases and eDNA specific features (persistence, transportation)	Depends on personal expertise and established ecological knowledge
Standardization	Standards need to be established	Standards already existing

3 eDNA in freshwater ecosystems

The pool of eDNA present in aquatic ecosystems originates from both microbial and macrobial organisms, including small-sized animals (zooplankton, benthic meiofauna). The interpretation of eDNA data may depend on its type and origin. In the case of microbial and meiofaunal components, DNA derived from organisms in environmental samples can be more directly related to the biology, occurrence and ecology of living organisms because whole individuals are present in eDNA samples. In the case of macrobial organisms, their DNA originates from the cellular remains suspended in water or bound to particles in the sediment. In this case, the detectability of eDNA depends on environmental and biological factors that are not related to the organisms themselves. These factors can be classified in three main categories: production, degradation, and transportation (Fig. 4). They have direct impact on detectability of macrobial eDNA, and to a lesser degree also on detectability of microbial and meiofaunal eDNA.

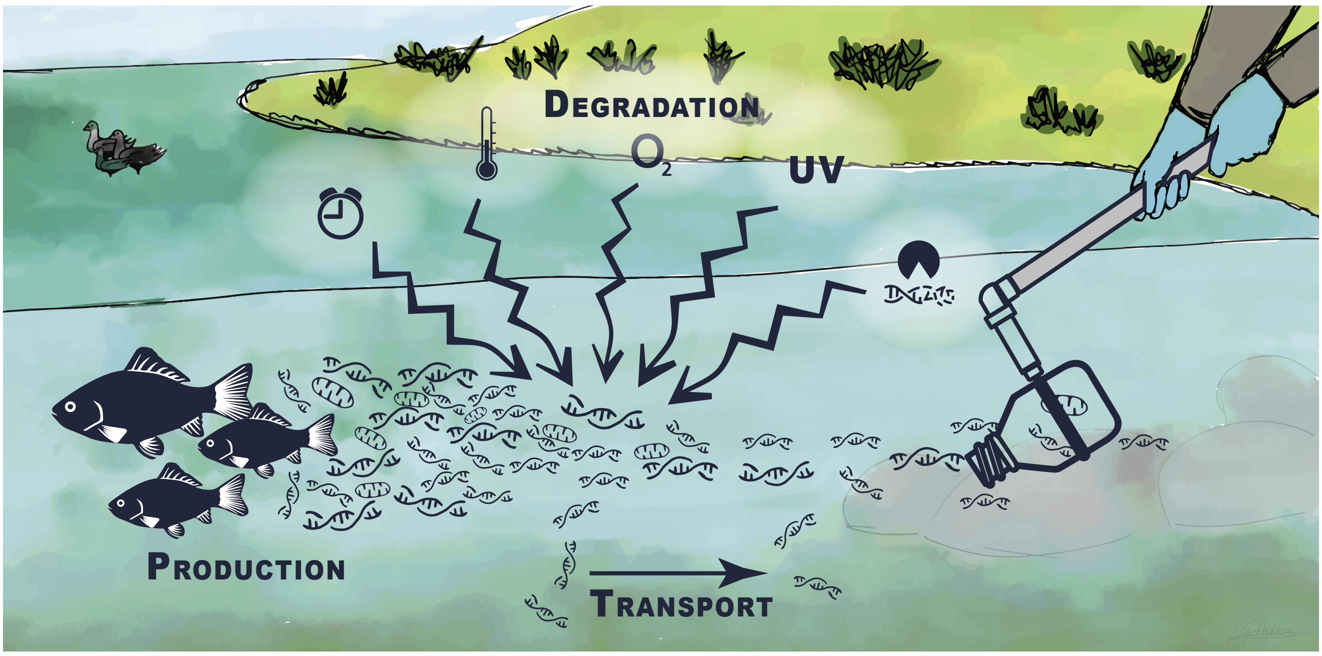
Production, that is the shedding of DNA into the environment, depends largely on abundance and density of a taxon and its biological and physiological features. Fish and amphibians are known to release large amounts of DNA to the environment, while arthropods release much less DNA, probably due to their exoskeleton. In general, the amount of released eDNA also depends on species-specific metabolic rates and can change during the life cycle, for example, increase during the breeding season (Maruyama et al., 2014; Bylemans et al., 2016). The variation in production of eDNA from different species can vary extensively in space and time and make quantitative interpretation of eDNA data difficult.

Degradation of eDNA depends on various physiochemical and biological factors, including temperature, UV, pH, ions and microbial activity (Strickler et al., 2015; reviewed in Barnes & Turner, 2016). Several studies show that macrobial eDNA persists longer in colder, darker and

Figure 4

The production and fate of macrobial eDNA in aquatic habitats

Production of eDNA occurs when organisms shed their DNA into their environment (e. g., fish releasing mucus). This eDNA is then subject to different degradation processes (temperature, microbial decomposition, etc.), and can be transported by passive flow in the water body. These three steps (production, degradation and transport) can affect the interpretation of eDNA data.



more alkaline conditions (Goldberg et al., 2015). Bacterial activity is also hypothesized to have strong impact on eDNA degradation, often in relation with physicochemical parameters such as temperature or demands for phosphorus. The obvious consequence of eDNA degradation is the reduced number of molecules that can be detected. It has been shown that the extra-organismal eDNA generally does not last more than 14 – 60 days in the water column (Goldberg et al., 2015). However, degradation can also lead to some chemical modifications of DNA molecules that can hamper the correct identification of species in eDNA data. Degradation of eDNA needs also to be considered after sampling and sample processing. For example, samples need to be stored and handled in ways that ensure the eDNA is preserved. This generally includes storing samples at – 20° C or in respective buffer solutions.

Transport of macrobial eDNA refers to the passive movement of intra-, extra-cellular or particle-bound DNA in the environment (e. g., by waterflow or wind), such that macrobial eDNA can be sampled at a different place than where it was produced. Transport has been mainly studied for lotic ecosystems. For example, it was estimated that eDNA can be transported over at least ten kilometers in streams (Deiner & Altermatt, 2014; Civade et al., 2016), and up to 100 kilometers in large rivers, with travelling time estimated at 41.7 hours for 100 km (Pont et al., 2018). It has been proposed that macrobial eDNA behaves as fine particle organic matter and its transport distance depends on hydraulic properties of the running water body (Pont et al., 2018). As eDNA can potentially be transported over long distances, its analysis provides biodiversity information at broad spatial scales and integrates information at the scale of (sub-)catchments (Deiner et al., 2016). Conversely, transportation can impede fine-scale interpretation of locations where a species actually occurs. For highly mobile species, like many fish, transport is less of a problem, but for habitat specialists this can pose challenges when interpreting eDNA results. Transport of macrobial eDNA is also driven by the movement of other species via what they eat and sub-sequentially excrete.

Detectability is the integration of production, degradation and transport, but also depends on sampling strategy. For example, the proximity of a sampled site to species habitat, or the volume of sampled material. Successful detection will also depend on molecular protocols, specifically efficiency of DNA extraction methods and the specificity of PCR primers.

3.1 eDNA study of different water bodies

The study of eDNA depends on the type of aquatic ecosystem studied. While there are some overall similarities in the sampling methods, there exists no standard eDNA sampling technique for all water body types due to the difference in their chemical and physical properties, and sampling methods need to be adapted to the type of water body investigated. The following chapter presents some eDNA characteristics specific to lentic and lotic ecosystems (summarized in Table 2).

3.1.1 Standing water bodies (lentic ecosystems)

Standing water bodies (especially ponds) were among the first water bodies sampled for eDNA detection of species (Ficetola et al., 2008), likely because of their small and well-defined size. Switzerland harbours many standing water bodies with more than 1500 lakes larger than 0.5 ha and even more ponds, but there is not an official and standardized method for biomonitoring of lakes or ponds. Their size is highly variable, and this may affect the macrobial eDNA and the way it can be sampled. Here, the focus is on the two extreme cases, small sized ponds and large lakes, knowing that a gradient between these two types exists in nature.

Ponds

Small ponds are less well documented and thus currently neglected in the regulatory framework in Switzerland. Ponds are also not considered under the European Water Framework Directive, likely because current sampling methods for other aquatic habitats are not suitable for them, thus the use of eDNA based monitoring in these systems could be transformational.

Ponds are highly variable in the water masses they contain and may in some cases even dry out periodically. Further, ponds are often stratified with little transportation of water in vertical or horizontal directions. This results in three important issues to consider when sampling eDNA from ponds (Harper et al., 2019a). First, based on stagnant water, macrobial eDNA is patchy in distribution and a representative sampling needs to include multiple samples taken across the pond. Second, the reduced flow leads to accumulation of DNA over time, but at the same time, temperatures of small water bodies are highly variable and especially elevated in summer, leading to faster degradation of eDNA. Third, pond systems are often characterized by a high turbidity, which often stems from organic dissolved materials or land run-off. The high turbidity poses challenges for filtration of water eDNA and the use of precipitation, larger pore size of the filters or a pre-filtration step may help to reduce this drawback. Organic dissolved materials can also seriously inhibit the PCR and hinder successful amplification in the laboratory downstream process. In pond systems, it is thus strongly recommended to use an internal positive control to quantify the occurrence of PCR inhibition.

Lakes

Switzerland is a country of many lakes and they are among the largest and deepest in continental Europe. The study of their diversity is strongly limited by accessibility (depths of 200 m and more) and size. However, size and depth of Swiss lakes vary substantially and influence the distribution of eDNA in the water body. Deeper lakes stratify in summer and winter, followed by a mixing phase in spring and autumn, respectively. Therefore, the seasonality in water movement will affect the distribution of the macrobial eDNA contained in the water column. It is important to take samples at different depths because some species, especially benthic fish, can only be found if samples are taken close to their habitat (Hänfling et al., 2016).

3.1.2 Running water bodies (lotic ecosystems)

Due to the distinct unidirectional flow of moving water bodies, like rivers or streams, the macrobial eDNA collected from water in these systems has a different spatial inference compared to standing aquatic ecosystems (Deiner & Altermatt, 2014; Deiner et al., 2015). The water movement transports eDNA through the system and is affected by discharge (Carraro et al., 2018). While this transportation processes (and the tightly and timely interlinked degradation) make eDNA approaches in running water bodies less useful for very localised assessments (at the point scale), this transportation offers the potential to infer catchment-level properties at the scale of up to several square kilometres (Deiner et al., 2016; Altermatt et al., 2020; Carraro et al., 2020).

Contrary to standing water bodies, which have a chronologically stratified and persistent sediment layer that allows reconstruction of biodiversity and environmental changes over past decades to centuries, the sediment is much more dynamic in lotic systems, is regularly stirred up, and thus has been less used for eDNA studies.

Streams

The eDNA found in streams can be affected by land-use surrounding the stream (Mansfeldt et al., 2020). The input from soils and leaves falling into streams not only leads to a terrestrial signal, but also leads to inhibition through humic acids, which complicates the use of PCR and requires the DNA to be purified further in the lab. Alpine streams fed by glacier receive many suspended solids and their seasonal regimes can vary tremendously between winter and snowmelt, while in low-land streams this variation can be neglected. At the same time, the density of wastewater treatment plants is increased in low-lands and sometimes their effluent feed large amounts of water into streams, leaving behind a trace of the wastewater community (Mansfeldt et al., 2020).

Rivers

With increasing size and volume of water flow, the rivers can present some specific challenges for detection of macrobial eDNA. Sampling from the shore may not give a representative sample and the sampling strategy may need to be adjusted to the organisms' habitat, and include samples taken in the middle and at the bottom of the river. For example, benthic fish species are more likely to be detected when water is sampled near the river bottom (Adrian-Kalchhauser & Burkhardt-Holm, 2016). It is discussed that larger volumes of water should be collected and filtered for rivers compared to streams or ponds (10 – 100 litres or more), however, this can be challenging due to sediments clogging the filters. The transport distance and deposition velocity of DNA containing particles must be taken in consideration for the interpretation of the data, as it can contribute to dispersal of eDNA over larger distance (Deiner & Altermatt, 2014).

3.1.3 Groundwater and springs

Groundwater is the most important source of drinking water in Switzerland. Groundwater is currently monitored in the program *Nationale Grundwasserbeobachtung* (NAQUA) (BAFU, 2019b), but no biological indicators are collected. Besides a handful of species-specific studies based on eDNA isolated from the groundwater, there is a limited number of publications characterizing microbial community from this habitat (Danielopol et al., 2000; Sohlberg et al., 2015), even though this method may be most suitable for a biological characterisation of groundwater habitats. Representative sampling can be challenging, as some types of groundwater can be hard to access, but drinking water wells may be a practical point of access. While the duration and spatial extent of water transportation in the underground is often unknown, the cold and dark environment is likely ideal to conserve eDNA.

Springs, on the other hand, are visible at the surface, but have hitherto largely been neglected in national monitoring programs. They offer habitats for highly specialized flora and fauna. Standardized methods to classify springs on the national level were recently implemented (Küry et al., 2019). eDNA metabarcoding might be particularly useful for their classifications, covering the broad range of organisms characterizing spring habitats (Amphibia, Turbellaria, Mollusca, Crustacea, Ephemeroptera, Plecoptera, Odonata, and Trichoptera, see Lubini et al., 2016). Springs and headwaters may also receive many inputs from terrestrial habitats, which likely results in an increased occurrence of eDNA from organisms of terrestrial origin.

Table 2

Selected specific aspects related to eDNA study of different water bodies

Ecosystem	Specific aspects	eDNA characteristics and possible mitigation solutions
Ponds	Stratification	Patchy distribution – multiple sites
	Reduced flow	Accumulation over time
	High turbidity	Filtration, PCR inhibition
	High temperatures	Faster degradation
	Drying out	Water eDNA not available
	Small area	High concentration of eDNA
Lakes	Size and seasonal stratification	Patchy distribution in time
	Depth specific habitats	Sampling different depth
	Algal blooms	Filtration, PCR inhibition
	Stratification of sediment	eDNA preserved over time
Streams	Transportation	Spatial inference depends on local movement of water body
	Input of organic material from soil and leaves	PCR inhibition through humic acids
	Downstream transportation	Integration of catchment
Rivers	Large size	Increased sampling volumes/specific sampling strategies
	Long distance transportation	Dispersal and spatial distribution
	Depth specific habitats	Surface and bottom sampling
	Downstream transportation	Integration of catchment
Groundwater	Can be difficult to access	Samples can only be taken at springs/groundwater wells
	Cold and dark environment	Good preservation
	Unknown transportation time and distance	Undefined origins and dynamics
	Poorly known biodiversity	Important gaps in DNA reference database
Springs	Cold temperature	DNA preservation
	Poorly known biodiversity	Gaps in reference database

3.2 Taxon-specific features related to eDNA study

The eDNA approaches have been applied to a wide range of taxonomic groups, focusing either on species' detection or whole-community surveys. There are several important questions that need to be considered when preparing an eDNA study with the focus on particular taxa:

- Are the taxa of interest well-represented in the environmental sample?
- What type of material should be sampled?
- Are sampling protocols available?
- What genetic markers and primer sets should be used?
- How complete is the database of the DNA reference barcodes?

Here, aspects specific to different taxonomic groups for their use in eDNA studies are presented and discussed (Table 3). In the appendix, detailed protocols and best practices are given for some of those.

3.2.1 Amphibians

Amphibians are highly suitable for eDNA studies because they are thought to shed substantial amounts of DNA into the environment, and can thus be detected relatively easily. Further, all species found in Switzerland are covered by the respective DNA reference databases. Amphibians were among the first groups of species to which macrobial eDNA approaches have been applied (Ficetola et al., 2008). There is extensive interest in using eDNA for this group because the detection through eDNA has been shown to be more sensitive and has lower false-negative rates than classic sampling procedures (Cruickshank et al., 2019).

eDNA is commonly used for the detection of specific amphibian species, such as the endangered great crested newt in UK (Biggs et al., 2015; Harper et al., 2017; Rees et al., 2014b). The eDNA detection of amphibians has been tested in streams and ponds, depending on the preferred habitat of the species of interest. The main source of amphibian eDNA is from the water, but the sampling techniques differ between studies. In pond habitats, the precipitation of eDNA is sometimes favoured due to suspended particles in the water column, but filtration can also be used (see chapter 4.1.1). Limitations of the approach exist in the case of species-complexes, such as water frogs of the genus *Pelophylax* or hybrids (for example *Triturus cristatus* and *carnifex*, in Switzerland), which cannot be told apart by eDNA. Also, species with semi-aquatic (*Salamandra salamandra*) or terrestrial (*S. atra* or *Hyla arborea*) lifestyles might be less often detected in water samples (Holderegger et al., 2019).

Genetic markers commonly used for detection of amphibian eDNA are mitochondrial 12S and 16S. There are specific 12S primers for frogs and salamanders (Valentini et al., 2016) and 12S primers for newts (Harper et al., 2018). The barcoding database is relatively complete for all European species.

3.2.2 Fish

Similar to amphibians, fish are suitable for detection using eDNA because they shed substantial amounts of DNA into the water, are relatively well covered in the databases, and traditional monitoring methods (especially electrofishing) are very resource-intense, invasive, and not applicable to large water bodies. The use of eDNA for fish species detection and inventory is thus more and more commonly applied in biomonitoring as an alternative to electrofishing or other invasive traditional methods (e.g., gill netting). Fish eDNA is not only found in the water column but is also present in sediments, where it can persist for longer time (Turner et al., 2014). Several studies indicate that benthic species are only detectable in samples collected in proximity to the specific habitat, for both lake and river systems (Adrian-Kalchhauser & Burkhardt-Holm, 2016; Hänfling et al., 2016). In large rivers, fish eDNA can be transported downstream over hundreds of km (Pont et al., 2018).

Genetic markers commonly used in fish eDNA metabarcoding are 12S and 16S. These markers are used because fish-specific primers (e.g., 12S MiFish primers, Miya et al., 2015) are available, which allow DNA-amplification and sequencing with a low proportion of non-target sequences. Unfortunately, these marker regions are not resolving recent speciation events, and thus cannot be used to tell species apart for some groups (e.g., *Coregonus* sp.). The COI barcode region, which is used for classic DNA barcoding (based on tissue samples), would have a slightly better (but still not complete) resolution, but has been largely abandoned for eDNA metabarcoding because of the lack of an appropriate fish-specific primer set. While the database of European (and Swiss) fish is mostly complete for the classical COI barcode (Geiger et al., 2014; Knebelsberger et al., 2015), there are still gaps in the 12S and 16S fish databases used for metabarcoding.

3.2.3 Mammals

Although monitoring of aquatic ecosystems is generally not focusing on mammals, their traces can be found in the water due to a semi-aquatic lifestyle or interactions with aquatic habitats. DNA from mammals may enter the aquatic ecosystem through their faeces, but also by direct contact (crossing aquatic ecosystems, drinking). While studies showed that mammals could be reliably detected in water eDNA from ponds in wildlife parks at which large mammals were drinking, it is more challenging to reliably and adequately record them in natural ecosystems (Harper et al., 2019b; Thomsen et al., 2012). The eDNA might be especially helpful for detection of small mammals such as water vole (*Arvicola terrestris*) that can be missed by camera traps (Harper et al., 2019b; Sales et al., 2019). Ushio et al. (2017) were able to detect a broad range of species (e.g., deer (*Cervus nippon*), mouse (*Mus musculus*), vole (*Myodes rufocanus*), raccoon (*Procyon lotor*), rat (*Rattus norvegicus*) and shrew (*Sorex unguiculatus*)) in water eDNA collected in a Japanese forest.

A species-specific assay for eDNA detection exists for the European otter (*Lutra lutra*, Thomsen et al., 2012). PCR primers for metabarcoding of mammalian species using 12S and 16S are available (MiMammal, Ushio et al., 2017; 12S-V5, Kitano et al., 2007; Riaz et al., 2011) and have been successfully used to detect mammalian DNA in water (e.g., Ushio et al., 2017; Harper et al., 2019b) and sediments (Sales et al., 2019).

3.2.4 Insects

Aquatic insects are commonly used to assess water quality in river systems, and therefore their monitoring using eDNA is of great interest. Aquatic insects, or arthropods in general, are covering a wide range of life cycles and are taxonomically very diverse. Thus, only selected groups have been used in past biodiversity monitoring (e.g., specific orders of insects). The source of DNA of insects (like other aquatic organisms) in the eDNA sample may vary widely, including faeces, mucus, gametes. It has been shown especially for lotic systems that such eDNA can be transported over large distances (e.g., Deiner & Altermatt, 2014), which may complicate comparison to classic samplings that are often very localised (kick-net sampling). Together with a relatively high level of uncertainty in classic sampling approaches, this makes comparisons to eDNA samples challenging. Many studies have analysed water eDNA to survey insect but the results of classical and molecular approaches were not always congruent (Fernández et al., 2018; Mächler et al., 2019). eDNA appears as a suitable method to cover the diversity of insects of larger subcatchments (i.e., for gamma diversity estimates), but less suitable for very localised analyses (Deiner et al., 2016). As such, the information gained by eDNA and classic methods may be of different spatial inference, and not directly comparable, but rather complementary. The biggest advantage of insect eDNA studies is the ability to sample across a much larger taxonomic range, including groups such as Diptera that are difficult to identify morphologically.

An alternative to using water eDNA to study insects is extracting DNA from the specimens collected in a kick-net sample and preserved as a bulk DNA tissue sample. Bulk DNA delivers more congruent results compared to classical water quality assessment techniques (Elbrecht et al., 2017). In this case, the kick-net sampling is performed according to the description of the module for macroinvertebrates (BAFU, 2019a) and the samples are preserved in molecular grade ethanol right on the field site. Further processing of these samples is done either on grinded tissues or DNA extracted from the preservative (Martins et al., 2019; Zizka et al., 2019). In both cases, insect species abundance is difficult to infer from metabarcoding data and species richness has been proposed as an alternative option (Beentjes et al., 2018; Buchner et al. 2019). While bulk DNA sampling and subsequent DNA extraction may result in datasets more comparable to the classic sampling, it does not reduce the work and invasiveness of the sampling associated with collecting the sample, and may transfer some limitations of classic approaches to a new technique (Blackman et al., 2019).

The recommended barcode region to be used for insects is highly debated. Generally used primers are based on 16S (Taberlet et al., 2018), 18S (Fernández et al., 2018) or COI (e. g., Leray et al., 2013; Geller et al., 2013; Elbrecht & Leese, 2017; Wangensteen et al., 2018) barcode regions. There is no consensus yet on neither the specific barcode region, nor the respective primers to be used. The COI barcode region is usually the preferred choice because of its better representation in DNA reference databases, especially for bulk samples. New, and more specific insects primers are currently under development.

3.2.5 Crustaceans

There is an especial interest in the detection of decapods (crayfish) (Krieg et al. 2019a), due to all native species being threatened by several invasive species and the pathogen they carry. Several eDNA studies use qPCR to detect single species of crayfish, but their results are controversial. In US lakes, *Orconectes rusticus* eDNA detection showed a good overlap with established sampling, but there was no good correlation to relative abundance (Dougherty et al., 2016). Other studies (e. g., on *Procambarus clarkia*, Tréguier et al., 2014) resulted in low accordance with established approaches especially when species abundance was low. Assays exist for the native noble crayfish (*Astacus astacus*, Agersnap et al., 2017; Krieg et al., 2019a) and the two invasive species, the signal crayfish (*Pacifastacus leniusculus*, Dunn et al., 2017; Mauvisseau et al., 2018; Krieg et al., 2019a) and the red swamp crayfish (*Procambarus clarkii*, Tréguier et al., 2014; Geerts et al., 2018; Mauvisseau et al., 2018; Riascos et al., 2018). Differences in organisms' seasonal activity seem to play an important role in the crayfish eDNA detection (Krieg et al., 2019a). Species-specific assays also exist for other crustacean species, such as amphipods and *Daphnia* (Egan et al., 2013; Deiner & Altermatt, 2014; Mächler et al., 2014).

Species-specific markers are predominantly designed for the COI barcoding region. Metabarcoding primers specifically designed and tested for crustaceans are not published, but crustacean eDNA can be detected with COI primers (e. g., Deiner et al., 2016; Blackman et al., 2017; Fernández et al., 2019). However, the results of the few water eDNA studies dealing with crustaceans are not conclusive, which may suggest that crustaceans shed relatively little DNA into the water.

3.2.6 Molluscs

Unlike crustaceans, molluscs tend to be easily detectable in water and sediment eDNA samples. Molluscs likely shed large amounts of DNA into the water (e. g., by mucus, and the filter feeding of mussels). Numerous studies proved the suitability of eDNA for the targeted detection of single species, and this approach is widely used to detect invasive zebra and quagga mussels (*Dreissena polymorpha* and *D. rostriformis bugensis*) in the United States and in Europe (Mahon et al., 2011; De Ventura et al., 2017; Gingera et al., 2017; Williams et al., 2017). Further eDNA assays for invasive species, such as *Potamopyrgus antipodarum* (Goldberg et al., 2013) and *Corbicula* sp. (Clusa et al., 2017; Cowart et al., 2018), are available. The eDNA assays also exist for endangered mollusc species, such as the swollen river mussel (*Unio tumidus*, Deiner & Altermatt, 2014) or the European pearl mussel (*Margaritifera margaritifera*, Stoeckle et al., 2016).

Specific molluscan metabarcoding primers are based on 16S (Klymus et al., 2017), but mollusc species can also be found with COI (Deiner et al., 2016; Fernández et al., 2018, 2019).

3.2.7 Oligochaetes

Aquatic oligochaetes are known to be sensitive to environmental changes and are recommended as excellent bioindicators of sediment ecological quality. However, their use in routine biomonitoring is impeded by the difficulties in their taxonomic identification based on morphological features. Recent studies investigate the possibility to analyse their taxonomic composition through metabarcoding of bulk samples or sediment eDNA. The results of these studies show that oligochaetes-based index of sediment quality is comparable to the index based on morphological study (see section 8.4.2).

3.2.8 Diatoms

The study of benthic diatom communities using eDNA is relatively advanced in Europe and its major aim is water quality assessment in rivers and streams (Kermarrec et al., 2014; Visco et al., 2015; Zimmerman et al., 2015; Apothéloz-Perret-Gentil et al., 2017; Vasselon et al., 2017a; Keck et al., 2018). The collection of the samples follows the sampling procedure of classic diatom monitoring: a representative subset of stones sufficiently submerged are collected, and the biofilm is removed with a single-use toothbrush, and stored in a buffer solution. Thus, the diatom DNA is not sampled from the water (as in most above examples), but from biofilm that cover the stones and contain living diatoms. In Switzerland, a Swiss Molecular Diatom Index is currently under development through the support of the NAWA campaigns (BAFU, 2013) as well as European project (SYNAQUA; see section 8.4.1; Lefrançois et al., 2017).

Two different markers are used to assess diatom communities, the nuclear 18S V4 region and the chloroplastic gene rbcL (Visco et al., 2015; Vasselon et al., 2017a). Most of European common diatom species are referenced in the rbcL database (Rimet et al., 2019).

3.2.9 Pathogens and parasites

Extensive work focuses on the detection of parasites and pathogens of aquatic organisms (mostly of fish, amphibians and crayfish; Krieg et al., 2019b). However, the detection of these parasites and pathogens is very time consuming by classic approaches, and eDNA approaches are seen as a valuable alternative, especially as most parasites have spore-like propagules that could be directly sampled from the water (Bass et al., 2015). For example, to detect the proliferative kidney disease (PKD), up to now fish had to be collected and dissected to track the parasite. eDNA offers a non-invasive approach to localize the pathogen without collecting the host, and even gives quantitative estimates of spore occurrence. Single-specific assays were developed to track agents for PKD (Carraro et al., 2018; Hutchins et al., 2018), freshwater saprolegniosis (Rocchi et al., 2016), crayfish plague (Strand et al., 2014; Robinson et al., 2018) and Chytridiomycosis (Kirshtein et al., 2007; Hyman & Collins, 2012). In Switzerland, those four diseases have an important environmental impact and specific assays are currently investigated (Krieg et al., 2019b). For fungi and oomycete species, the markers used are ITS or 18S, whereas COI is used for *Tetracapsuloides bryosalmonae* (PKD agent), which is a cnidarian species.

3.2.10 Aquatic plants (macrophytes and phytoplankton)

Plants are a further group of organisms monitored in aquatic ecosystems, with macrophytes being both monitored in lentic and lotic systems, and phytoplankton being only monitored in lentic system. While there is a general interest in both groups, relatively few standardized monitoring tools exist even for classic approaches (Känel et al., 2017). Some studies designed species-specific primers for invasive species such as *Myrophyllum aquaticum* (Scriver et al., 2015), *Elodea densa* (Fujiwara et al., 2016), *E. canadensis* and *E. nuttallii* (Gantz et al., 2018), or *Hydrilla verticillata* (Matsuhashi et al., 2016; Gantz et al., 2018).

Macrophytes, and plants in general, need multiple loci to get a sufficient resolution both at higher as well as at lower taxonomic levels (Hollingsworth et al., 2011). The use of multiple markers, however, is challenging or even impossible because the different marker regions looked at cannot be assigned to individual organisms any more. Nevertheless, promising regions for plant metabarcoding primers are rbcL, ITS2 (Fahner et al., 2016; Kuzmina et al., 2018) or trnL (Taberlet et al., 2007), but species resolution might be limited. Alternatively, barcode regions such as matK, trnL might be reasonable for species-specific detections (e. g., Scriver et al., 2015; Matsuhashi et al., 2016). Community analysis of phytoplankton using eDNA are very sparse, but the chloroplastic 23S gene seems to be suitable for assessing diversity (Cannon et al., 2016; Craine et al., 2018).

Table 3

Examples of published eDNA studies for selected taxonomic groups

Taxon	Target species or group	Potential application	Method	References
Amphibians	Bull frog (*Rana catesbeiana*)	Invasive alien species (IAS) detection	PCR	Ficetola et al., 2008
	Great Crested Newt (*Triturus cristatus*)	Monitoring of endangered species	qPCR Metabarcoding	Rees et al., 2014b; Biggs et al., 2015; Harper et al., 2017, 2018; Buxton et al., 2018
	Smooth newt (*Lissotriton vulgaris*)	Monitoring of endangered species	qPCR Metabarcoding	Smart et al., 2015; Charvoz, 2019
	Fire salamander (*Salamandra salamandra*)	Monitoring of endangered species	qPCR	Preissler et al., 2018
		Total biodiversity survey	Metabarcoding	Valentini et al., 2016
Fish	Round goby (*Neogobius melanosomus*)	IAS detection	PCR qPCR	Adrian-Kalchhauser & Burkhardt-Holm, 2016; Nevers et al., 2018
	European eel (*Anguilla anguilla*)	Monitoring of endangered species	qPCR	Seymour et al., 2018
	Silver carp (*Hypophthalmichthys molitrix*)	IAS detection	qPCR	Amberg et al., 2015; Erickson et al., 2017
	European weather loach (*Misgurnus fossilis*)	Monitoring of endangered species	qPCR	Sigsgaard et al., 2015
		Total biodiversity survey	Metabarcoding	Hänfling et al. 2016; Pont et al., 2018
Mammals	European otter (*Lutra lutra*)	Monitoring of endangered species	qPCR	Thomsen et al., 2012
		Total biodiversity survey	Metabarcoding	Harper et al., 2019b; Sales et al., 2019
Aquatic insects	Asian tiger and bush mosquitoes (*Aedes albopictus*, *A. japonicus japonicus*)	Disease vector detection	qPCR	Schneider et al., 2016
	Large white-faced darter (*Leucorrhinia pectoralis*)	Monitoring of endangered species	qPCR	Thomsen et al., 2012
	Ephemeroptera, Plecoptera, and Trichoptera	Total biodiversity survey	Metabarcoding	Hajibabaei et al., 2011; Mächler et al., 2019
	Chironomidae	Total biodiversity survey	Metabarcoding	Carew et al., 2013; Bista et al., 2017
		Total biodiversity survey	Metabarcoding	Deiner et al., 2016; Fernández et al., 2018; Macher et al., 2018
		Biotic index	Metabarcoding	Elbrecht et al., 2017

Taxon	Target species or group	Potential application	Method	References
Crustaceans	Rusty crayfish (*Orconectes rusticus*)	IAS detection	qPCR	Dougherty et al., 2016
	Signal crayfish (*Pacifastacus leniusculus*)	IAS detection	qPCR	Dunn et al., 2017; Mauvisseau et al., 2018
	Red swamp crayfish (*Procambarus clarkii*)	IAS detection	qPCR	Tréguier et al., 2014; Riascos et al., 2018
	Noble crayfish (*Astacus astacus*)	Monitoring of endangered species	qPCR	Agersnap et al., 2017
Molluscs	Zebra mussel (*Dreissena polymorpha*) Quagga mussel (*D. bugensis*)	IAS detection	PCR qPCR	Egan et al., 2015; De Ventura et al., 2017
	New Zealand mud snail (*Potamopyrgus antipodarum*)	IAS detection	PCR qPCR	Clusa et al., 2016; Goldberg et al., 2013
	Asian clam (*Corbicula fluminea*)	IAS detection	PCR qPCR	Clusa et al., 2017; Cowart et al., 2018
	Chinese pond mussel (*Sinanodonta woodiana*)	IAS detection	PCR	Clusa et al., 2017
		Total biodiversity survey	Metabarcoding	Klymus et al., 2017
Oligochaetes		Total biodiversity survey	Metabarcoding	Weigand & Macher, 2018
	Aquatic species	Biotic index	Metabarcoding	Vivien et al., 2019
Diatoms	Benthic species in rivers and streams	Biotic index	Metabarcoding	Visco et al., 2015; Apothéloz-Perret-Gentil et al., 2017; Vasselon et al., 2017a
Pathogens and parasites	*Tetracapsuloides bryosalmonae*	Detection of Proliferative kidney disease agent	qPCR	Carraro et al., 2018; Hutchins et al., 2018
	Saprolegnia parasitica	Detection of Freshwater saprolegniosis agent	qPCR	Rocchi et al., 2016
	Aphanomyces astaci	Detection of Crayfish plague agent	qPCR	Strand et al., 2014; Robinson et al., 2018
	Batrachochytrium dendrobatidis	Detection of Chytridiomycosis agent	qPCR	Kirshtein et al., 2007; Hyman & Collins, 2012
Aquatic plants	Waterweeds (*Elodea* spp.)	IAS detection	qPCR	Gantz et al., 2018
	Phytoplankton	Total biodiversity survey	Metabarcoding	Cannon et al., 2016; Craine et al., 2018

4 Sampling for eDNA analysis

4.1 Types of environmental DNA source material

The choice of eDNA method used will depend on the type of ecosystem and the taxonomic group investigated. Different habitats and taxa require different types of samples and different protocols (Table 4). Overall, there are four types of environmental samples from which DNA can be isolated for aquatic biomonitoring:

- Water (section 4.1.1)
- Sediment (section 4.1.2)
- Biofilm (section 4.1.3)
- Bulk macroinvertebrate DNA (section 4.1.4)

Table 4

The sources of (e)DNA ranked depending on taxonomic groups

The preferential use of different sources for detection is indicated by signs with the following meaning: +++ preferred source, ++ good source, + moderate source, and – not ideal source. Please note that future method development may change this interpretation, so current literature should always be assessed

Taxa	Water	Sediment	Biofilm	Bulk
Amphibians	+++	+	–	–
Fish	+++	+	–	–
Mammals	+++	+	–	–
Aquatic Insects	++	++	–	+++
Crustaceans	+	+	–	+++
Molluscs	+++	+++	–	++
Oligochaetes	+	++	–	+++
Diatoms	+	+	+++	–
Pathogens and parasites	+++	+	–	–
Macrophytes and phytoplankton	+++	+	–	–

Only the most important bioindicator taxa are included here. In the appendix, detailed protocols and best practices are given for some of those. However, eDNA can also be used to analyse a wider diversity of meiofauna, zooplankton, fungi, and various microorganisms. For example, one could use eDNA to assess the diversity and composition of protists such as ciliates, or rotifers, which may be very good indicators of the environmental state of a system, and which have not, or hardly, been used in classic biomonitoring due to the lack of expertise and methods available. Some of these groups of organisms may require specific protocols that are not included here, but the general principles will not differ much.

4.1.1 Water eDNA

To collect eDNA from water samples, there are two common techniques used:

- Filtration
- Precipitation

Filtration is often favoured over precipitation due to the possibility to handle larger amounts of water. However, there are certain situations where precipitation may be more suitable.

Filtration

Filtration collects the DNA on the filter matrix; mainly DNA that is still in cells, organelles or bound to particles. Currently, several filtration methods are published, with no single best method identified. The methods differ by the filter material and technique. The main questions to be asked when preparing filtration are:

- What kind of filtration equipment shall be used?

There are various forms of filtration techniques possible, including hand filtration, as well as filtration using peristaltic or vacuum pump (Fig. 5). The hand filtration uses minimal material (a syringe and a filter) and is therefore easy to be conducted also at remote sampling sites. The simplest filtration procedure is done using disposable syringes (usually 50 mL or 100 mL syringes) and respective filters. However, depending on pore size and suspended particles in the water body, this requires some physical strength. Silicon guns can be used to do the filtration. As an alternative, a peristaltic pump is often used when

Figure 5

Three types of filtration to capture eDNA from water

A filter capsule directly mounted on a syringe (left), a peristaltic pump (middle), and a manual vacuum pump (right).

Middle Photo: Eawag, Peter Penicka

filtering larger volumes (i.e., several litres). It needs less physical strength for filtration, but the pump is usually run by a car battery, which is difficult to carry to remote sites. Between sites, all tubes of the peristaltic pump need to be replaced in order to reduce cross-contaminations. Finally, vacuum pumps can be used to filter small to large volumes. They are efficient, but require more complex equipment, and tubes and filter cups need to be replaced between sampling sites. Vacuum pumps can be operated both in the lab and in the field.

- What would be the most appropriate filter pore size?
- Is the use of encapsulated or open filters recommended?

The most widely chosen filters are membrane filters, and the specific products chosen are reflecting commercially available types, with eDNA-specific filters being currently developed. The pore size of a filter can determine what kind of eDNA will be collected. Pore sizes between 0.22 and 0.7 µm are mostly used in eDNA studies. Using smaller pore size (e.g., 0.22 µm) allows capturing most cells and organelles and is usually used for the detection of micro-organismal DNA. However, there is a trade-off with smaller pore sizes and the amount of water that needs to be filtered before the filter clogs. This can be partly solved by using filters with a larger diameter. Contamination issues often occur with filters that arrive open and need to be placed in a filter housing or in the specific set-up of a filtration system. Currently, the use of encapsulated filters, such as Sterivex® or analogue products, are the preferred option, because of the easy handling and reduced risk of contamination.

- Shall the filtration be done in the field or in the lab?

Generally, filtration can be carried out in the field or in the lab. It is better to filter directly in the field, as the risk of cross-contamination is lower, and transportation of the samples is easier (cooled to < 5° C for a few hours, or stored in Longmire's lysis buffer). Filtration in the lab may allow large volumes to be filtered (especially for filtration techniques that need electrical pumps), but is only feasible when the lab facilities can be reached within a short amount of time (max. few hours), in order to avoid degradation of macrobial eDNA or changes in microbial communities. Filtering in the lab also requires greater precaution, as all samples are handled at the same place and additional actions are needed to minimize cross contamination.

- How much water to be sampled for filtration?

Generally, the more water is sampled, the more likely it is to detect a species. However, the amount of possible PCR inhibitors also increases with the volume of water filtered, and filtering larger volumes is logistically challenging. Thus, the volume filtered is a pragmatic decision, which may depend on the type of environment.

For streams, it is best to collect water from multiple sites from the shore. So far, most of the studies filtering eDNA from streams collected between 0.5 – 2 L of water, which is often sufficient to recover large parts of the diversity. For rivers and lakes, and especially to detect rare species, most studies have been filtering between 1 to 100 L per site. However, collecting this large amount is only feasible with peristaltic pumps and not with syringe-based filtration. Given that temperate habitats are less diverse, the current practice of filtering around 2 L consisting of multiple sub-samples from a given site seems to be workable.

Precipitation
While filtration is often favoured over precipitation due to the ability to process larger volumes and no handling of chemicals in the field, all filtration techniques are affected by suspended particles, which is less of an issue for precipitation, making the latter technique advantageous in some cases. Suspended particles do not interfere with the extraction. The principle of precipitation is to use a salt and ethanol mix to precipitate the DNA/RNA contained in the water. This mix is then centrifuged, to collect the pellet containing the DNA. As most centrifuges are designed for small tubes, precipitation is often restricting to a total volume of 50 mL. This limitation can be bypassed by collecting multiple samples, but it may still be not feasible to collect large volumes, and is (except for ponds) not currently the method of choice.

Precipitation is commonly used for detecting amphibians in ponds. Biggs et al. (2015) showed that 20 sub-samples of 30 mL need to be taken to get close to 100% detection rate for the great crested newt in ponds. Natural England advises eDNA collectors for the great crested newt sample 20 × 30 mL, mix the sub-samples and thereof take 6 × 15 mL (this is recommended for ponds that are about the size of 1 ha). In Switzerland, eDNA metabarcoding of amphibians using precipitated eDNA is commercially offered (see Holderegger et al., 2019). The suggested number of samples increases with pond size, and the number of 50 mL samples to be taken is 3 – 5 for ponds < 50 m^2, 6 – 10 for ponds of 50 – 500 m^2, and 10 – 20 for ponds > 500 m^2. All sub-samples are mixed and finally, 3 × 15 mL of this mix are used for the precipitation step. However, other authors suggest taking less subsamples but greater volume for each (see Hänfling et al. 2016).

4.1.2 Sediment eDNA
Complex organic and inorganic particles in the sediment can bind and stabilize DNA. Therefore, the preservation time of DNA in sediment is extended compared to water. This creates the opportunity to go back in time by sampling a sediment core that has archived DNA over centuries to millennia (Monchamp et al., 2018).

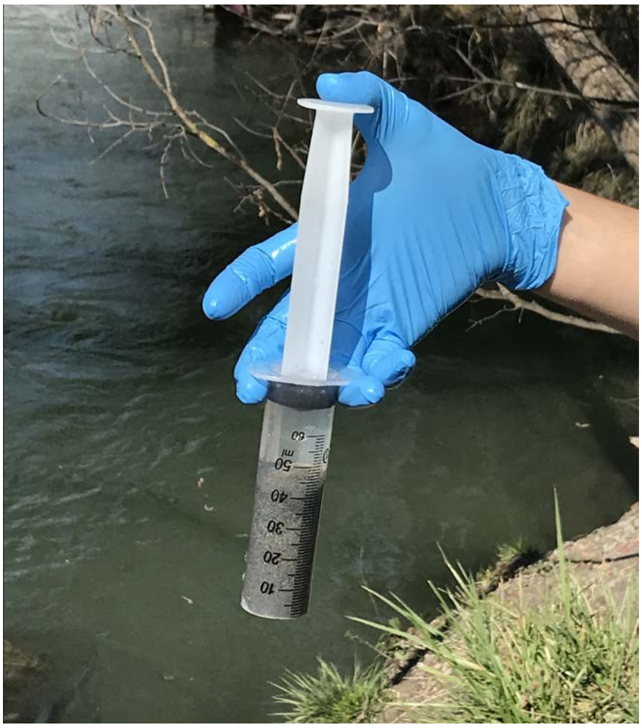
Sediment sampling is preferentially done in deep lakes, where sediments can settle and are not constantly stirred up. However, sediment eDNA can also be an important source of information about the taxonomic composition of meiofauna (nematodes, oligochaetes) and protists (e. g., ciliates) that are used as bioindicators of organic enrichment and other environmental impacts in large rivers or lakes.

The sampling material used for collecting sediment eDNA depends on the depth and accessibility of sampling sites (Fig. 6). Deep lake sediment sampling is usually done with heavy equipment, such as corers or grab samplers. The coastal sediment samples can be collected by spoons (single-use), or by coring using a syringe with the end cut off. The later method allows precise volumetric samples of sediment layers.

The volume of sediment sampled depends on the extraction method. The commercial kits are adapted to a maximum 5 – 10 g of sediment. Most available sediment/soil kits are for microorganisms and can only process 0.2 – 0.5 g samples. The manual processing of sediment samples is also limited by the size of centrifuge tubes. Preserving sediment samples in ethanol prior to DNA extraction is also possible and commonly used in microbial analyses (Lanzén et al., 2017). However, the simplest method is to store sediment samples at – 20° C.

Figure 6

Core sample of near-shore sites can be taken with a single-use syringe with the tip being cut off



4.1.3 Biofilm eDNA

Epilithic biofilm is formed at the surface of the stones by bacteria and unicellular algae. These biofilms are in direct contact with the water, and therefore the community forming the biofilm responds directly to the changes in the water quality. Moreover, compared to sediment, only a small proportion of dead organisms stay in place and most of them are washed away by the water current. At present, biofilm samples are only used to target living diatoms in running waters in order to infer biological quality index. Methodology to sample biofilm for diatoms is detailed in the diatom module for streams bioindication (Hürlimann & Niederhauser, 2007) and summarized in the diatom case example (section 8.4.1). This method is well standardized and also used and accepted in other European countries.

4.1.4 Bulk macroinvertebrate DNA

Bulk sampling involves the collection of specimens using a classical sampling procedure (e. g., a kick-net sample for macroinvertebrates according to the relevant Modul-Stufen-Konzept, Stucki, 2010; BAFU, 2019a) and subsequent DNA extraction of the homogenized specimens or the molecular grade ethanol used to preserve the sample. This approach has especially been used for macroinvertebrates, in particular aquatic insects. The advantage of bulk DNA study is the rather exact spatial coverage and the lower interference with chemical or physical properties of the sampled environment. The disadvantage is that the sampling procedure (collecting and pre-sorting invertebrates) cannot be automated, is time-consuming, and has the same limitations on specificity and repeatability as the classic sampling (Blackman et al., 2019).

There are two different routes to get DNA from a bulk sample. Either the DNA is directly extracted from the specimen tissues or it is extracted from the preservative solution (ethanol) the sample has been placed in. The extraction from tissues requires sorting of the individuals from the debris, which can be time consuming and may induce the errors through overlooking of some small specimens or larval stages. Next, the tissues of the individuals need to be dried and homogenized before the DNA is extracted from this tissue mix.

The extraction of DNA from the preservative solution is more straightforward, and it is based on the fact that the specimens stored in ethanol release their DNA into the ethanol, which can then be captured by filtering or precipitation. This is a relatively novel and highly promising solution, but not many scientific results are available yet (Zizka et al., 2019). So far, most studies used 80% or higher concentrations of molecular grade ethanol to preserve samples for a few weeks prior to filtration. Storing in formaldehyde or stains, like Rose Bengal, must be avoided, as they interfere and prohibit the routine use of this method, although rapid fixation in formalin prior to specimens sorting was possible in the case of oligochaetes (Vivien et al. 2016). The persistence of whole individuals for further morphological analyses and the omission of a primary sorting step are the main advantages of using preservatives as a source of DNA for metabarcoding analyses.

4.2 Precautions for handling eDNA samples

Laboratory methods for the detection of eDNA are optimized to discover small traces of DNA and therefore these techniques are extremely susceptible for contaminations. Practitioners need to be aware of this risk and need to take several actions at different sampling steps to minimize the probability to contaminate the samples (Fig. 7).

Material

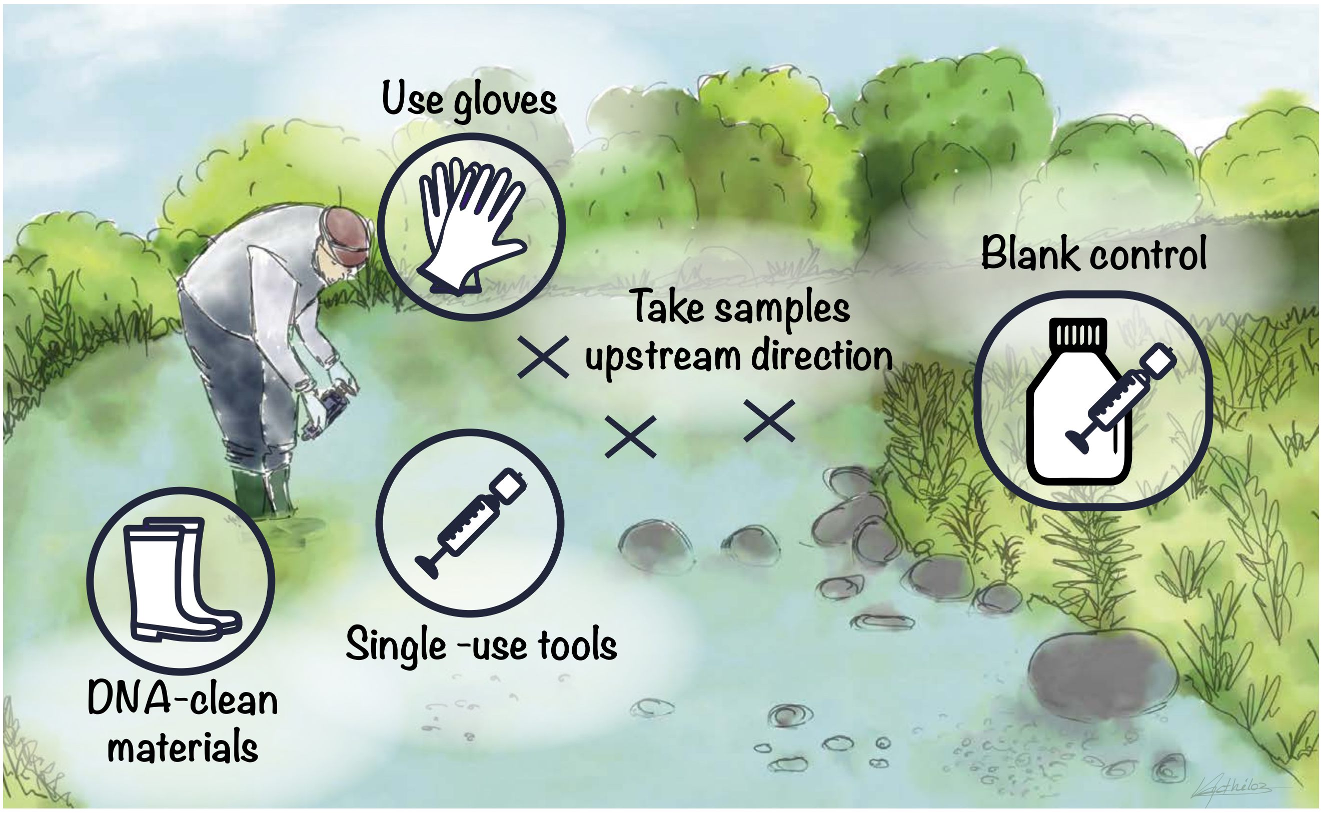
The general principle is that all material and equipment that gets in contact with the eDNA sample must be either of single-use or cleaned to be DNA-free. Best practices are to wear single-use disposable gloves during sampling. This not only prohibits contamination by human DNA, which can possibly interfere and dominate the sequencing output, but will also reduce cross-contamination between sampling sites.

Sealed, single-use tools (like syringes or spoons) that are opened and used at a given site are recommended. However, some tools may be too expensive to be single-use only and therefore need to be reused at multiple sites. Reusable material needs to be cleaned between the different sites. The best cleaning option is sodium hypochlorite (bleach, 5 – 10% solution). However, large volumes of bleach should not be used in the field. Commercial products, such as 'DNA away' or 'DNA-Exitus Plus' are decontamination solutions that are safe to use in the field but are more expensive. Rinsing with ethanol or water (even deionised water) is not sufficient to decontaminate; it may reduce some cross-contaminations due to dilution effects, but it is not breaking down any DNA. However, it is recommended to rinse cleaned equipment with ddH_2O after cleaning and before collection of eDNA samples to remove any bleach or detergent.

Figure 7

Measures to avoid contamination during sampling

All equipment must be cleaned of any DNA, single-use gloves and tools must be used to prevent contaminations, while blank control samples can be used to detect potential contaminations.



Controls
The biggest concern of all eDNA studies are false positive and false negative records. The first is the detection of an eDNA signal in absence of the organism and/or its DNA in the environment; the latter is the lack of an eDNA signal in presence of the organism and/or its DNA in the environment. False positives can occur due to contaminations at the sampling, extraction, and sequencing step. False negatives can occur due to failing extraction, PCR or sequencing steps or subsampling effects. To exclude false positives/false negatives, or to at least know about their occurrence, controls and replication are an important part of all eDNA studies.

Controls allow the identification of contaminations and should be included at all steps of the sampling and analysis. This is especially needed when equipment that comes in close contact with the sample (e. g., filter housings, silicon tubes) is reused. In water eDNA sampling, the use of a blank control consisting of DNA-free water, for example ultrapure water treated with UV light, is a well-established practice. Unfortunately, efficient UV equipment is hardly available in laboratories outside academia and therefore the use of commercially available mineral or deionized water can be used as a simplified negative control. Controls should be implemented at the start and the end of each eDNA sampling campaign as a minimum. This would, for example, include the filtering of DNA-free water in the field (at a sampling site) at the beginning and the end of each sampling day. As a minimum, negative controls can be collected at the first step and processed along with the field samples for the whole workflow. Importantly, the controls must be taken following the same protocol as the true samples, except that the water filtered (or precipitated) is DNA-free, and not an environmental water sample. The negative control should also subsequently be processed along the same procedure as all other samples. Usually no controls are implemented in the case of sediment and biofilm sampling.

Sampling scheme
Going to the field requires some planning, especially when visiting multiple sites at the same time. Ideally, the water body itself should not be entered during sampling. However, if this is not possible, there are two options. Firstly, all material (waders, etc.) that is in contact with the sampled environment needs to be cleaned as explained above. Secondly, sampling should be performed in a way to minimize contaminations. In rivers for example, sites should be visited in an upstream direction starting with the site at the lowest point of the catchment to avoid mobilising DNA higher in the catchment. Similarly, samples should be collected upstream of the person standing in or close to the water, such that the water sampled has not been in contact/passed the person or equipment.

4.3 Other technical issues related to eDNA sampling

- How many eDNA replicates shall be taken?

Multiple replicates are usually used to ensure the reliability of eDNA data. For example, when a species is detected in multiple replicates it is more likely that this species is truly present in the environment and it is not a false positive. To be statistically valid, a sampling campaign should thus include multiple independent replicates per sampling site, and eDNA studies have shown that some level of replication is needed for an appropriate interpretation (e. g., Mächler et al., 2019). However, performing replication is time-consuming and costly, and these costs need to be traded-off with the benefit. In many cases, three independent replicates per site are used as the minimal level of replication. In sites with very patchy eDNA distributions, such as ponds, or very large water bodies, such as lakes, more subsamples and replicates are needed. Number of replicates can also depend on the target of the study (community survey or rare species detection). Replication at the sampling level can be done either by pooling before the DNA extraction (usually cheaper, but less recommended), or with no pooling until the sequencing has been done, such that all replicates are sequenced independently (in a random order). The latter is recommended and allows to estimate detection thresholds at the per-sample base.

- What is the best way to transport and store eDNA samples?

When not properly stored, eDNA can degrade relatively quickly in water samples or on filters due to microbial activity. Thus, the best practice is to extract it immediately after sampling. However, this is not always possible and thus samples need to be transported and often stored over a longer period. Coolers must be used for sample transportation, and eDNA samples should not be exposed to warm temperatures (e. g., occurring in a car in summer). Freezing at −20° C is best for filters, precipitated water, sediment and even the water itself. It is required to have a constant temperature and thawing/freezing events need to be strictly avoided. If the sample is directly transferred to a preservative or a buffer (as for example from biofilms or bulk), then the sample can be transported and stored at 5° C or even at (maximally) room temperature over a longer period of time as the buffer/preservatives stabilizes the DNA. Nevertheless, it is always recommended to store those samples at least in the refrigerator or cool room to slow down the process of DNA degradation. Filters can also be dried with silica beads and a desiccator, but this method has not yet been properly tested.

- When and where should the samples be taken?

The sampled microhabitats should be selected according to the most-likely occurrence of the species. For example, eDNA sampled at the bottom instead of the surface for increasing detectability of species that are bottom dweller (Adrian-Kalchhauser & Burkhardt-Holm, 2016). Sampling in a period of hibernation and inactive stages should be avoided (e. g., during hibernation, De Souza et al., 2016).

5 Molecular lab

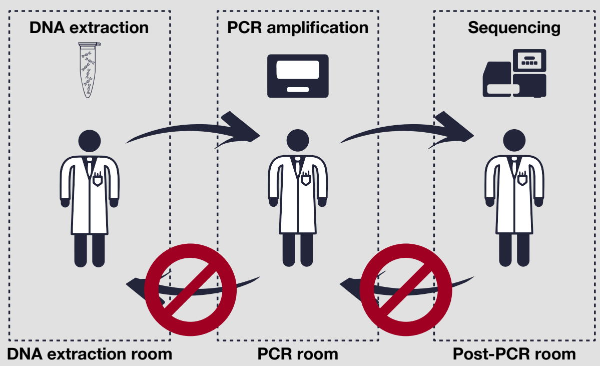
When analysing samples of eDNA, low to very low amounts of DNA are studied. To prevent erroneous results due to contaminations, all laboratory work must be done following strict protocols and practices. In the following part, the workflow and individual laboratory steps are described. This must be done in a laboratory environment specifically dedicated to the eDNA work, and needs to be complemented with adequate negative and positive controls. The recommended practices include a separation of pre- and post-PCR steps (eDNA extraction and PCR must be done in separate rooms with a unidirectional workflow), the use of protective clothing and equipment (sterile bench). The laboratory infrastructure and equipment must be dedicated to the specific working steps, and need a predefined cleaning procedure. Ideally, the clean lab has a positive air pressure to reduce contaminations (Fig. 9).

Important precautions for the wet lab work

Figure 8

A unidirectional workflow must be applied to all laboratory steps

DNA extraction must be physically separate from all subsequent steps, and neither material nor persons can go in the reverse direction.



Dedicated post-PCR room

Since the concentration of target DNA in a sample can be very low, especially in water samples, it is very important to be vigilant to avoid contamination. In addition to common best practices (working carefully, wearing a lab coat), some special precautions must be taken when working with eDNA. Most importantly, pre-PCR work and all equipment associated to it must be physically separated from post-PCR work. Therefore, the DNA extraction and the preparation of PCR must be done in rooms separate from the post-PCR process. PCR machines should be placed either in the post-PCR room or in a dedicated room.

Unidirectional workflow

A unidirectional workflow from low to high DNA concentration should be followed to mitigate contamination with amplified DNA from previous assays (Fig. 8). This rule is true for both people and material. Consequently, laboratory staff must absolutely avoid going from high DNA concentration to lower DNA concentration rooms on the same days. One must not go back to work in the extraction room after working on the post-PCR process on the same day, nor to bring PCR reaction products to the pre-PCR labs (even for PCR positive control). Ideally, there are two pre-PCR eDNA rooms to separate working with "high" or "low" DNA levels (for example separating bulk extractions from water and sediment samples).

Single-use material

All consumables used at different steps must be specific to one application. One of the most important sources of contamination are the micropipettes. Therefore, it is very important to have at least one set of pipets for each room ("low" DNA extraction, "high" DNA extraction, PCR set-up, specific pipet to add the DNA to the PCR reactions) as well as using filter tips in all steps of the process. The same rule applied to the equipment, which has to be specific for their application (e.g., centrifuge, racks, and fridge). Negative controls during pre-PCR and PCR steps are required to ensure proper material and handling. Importantly, all material, equipment and working places have to be decontaminated after each lab session with the use of UV light or bleach solution.

Figure 9

Clean lab used for extracting eDNA

The clean lab (left panel) must be physically separate from rooms in which post-PCR products are handled. It should have positive air pressure, walls and furniture need to be cleaned regularly, and access is restricted to trained personnel. Working in the lab (right panel) is done using protective laboratory clothing and in sterile conditions, to avoid contaminations of the samples.

Photos: Eawag, Roman Alther

5.1 General workflow

The standard eDNA study consists of several steps that involve processing of environmental samples (water, sediment, biofilm) or bulk samples to obtain genetic information about organisms present in those samples or their DNA traces.

The processing of eDNA samples can be divided into three wet lab steps, which comprise:

1. **DNA extraction** – The molecules of DNA (extracellular DNA and DNA of living organisms, their cells and organelles) are isolated from environmental samples.
2. **Polymerase Chain Reaction (PCR) amplification** – multiple copies of a targeted genomic region are produced in a series of enzymatic reactions.
3. **High-throughput Sequencing (HTS)** – The PCR-amplified products are used as template for massively parallel DNA sequencing producing millions of sequences (this step concerns metabarcoding only).

The methods applied to process eDNA samples will depend on the aim of the ecological study or bioassessment purpose (Fig. 10).

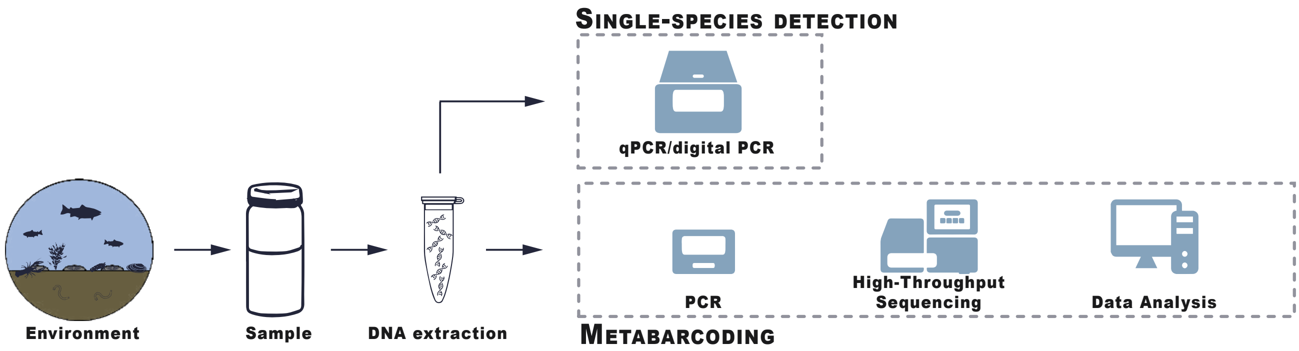
- If the aim is **to detect a single species** of endangered or invasive taxa, parasite or pathogen – the most appropriate approach is using conventional PCR, quantitative PCR (qPCR) or digital PCR (dPCR) methods (section 5.4)
- If the aim is **to analyse community composition** for a biodiversity survey or ecological quality assessment – the most efficient approach is to use metabarcoding, that is, high-throughput amplicon sequencing (section 5.5)

In both cases, the eDNA sampling and DNA extraction follow the same protocols. The same eDNA samples can be used for both species detection and biodiversity surveys. However, all steps following the DNA extraction, including molecular lab and data analysis are different.

The choice of method also depends on access to the equipment and expertise. PCR-based single-species detection methods are faster, less expensive and easier, while HTS-based metabarcoding requires more specialized equipment and higher-level expertise in generating and interpreting sequence data. Their advantage of getting information about multiple species at a time is an important consideration when planning a biomonitoring study, and the single-species detection is only advisable for a few focal organisms (e.g., rare or invasive species, targeted pathogens) with well validated single-species primers.

Figure 10

Workflow of eDNA analysis for single-species detection and metabarcoding



5.2 DNA extraction

The way DNA is extracted is highly dependent on the type of the sample, but the general workflow is always the same. The first step consists in the lysis of sampled material in order to isolate DNA present in cells or organelles (mitochondria or chloroplasts). The lysis can be performed either using chemical components present in the lysis buffer, or through mechanical disruption of the walls and membranes, usually by adding beads to the sample and shake it with the help of a vortex or a bead-beater. During the second step, all organic and inorganic components except DNA are removed. As some types of environmental sample are known to contain PCR inhibitors, this step usually includes removal of such inhibitors to ensure a proper DNA amplification. Finally, the DNA extract is purified either on silica membrane, with magnetic beads or through precipitation.

Several branded kits are available on the market for different type of DNA-extraction and can be used to extract eDNA from water samples (e.g., Qiagen, Macherey-Nagel). Furthermore, homemade protocols, mainly based on phenol-chloroform extraction, exist. Common methods used for collection and extraction are reviewed by Tsuji et al. (2019). Although the extraction methods are not considered as a limiting factor in some specific cases (Vasselon et al., 2017b), using different extraction methods can result in significantly different species assemblages (Deiner et al., 2015, 2018). Therefore, a consistent use of the same protocol during one project is necessary to ensure the reproducibility and comparability of the results.

5.3 PCR amplification

Principle

The amount of target DNA present in environmental sample is relatively low. It is therefore necessary to amplify the target DNA barcode region using the polymerase chain reaction (PCR) before sequencing. The principle of this method is to generate multiple copies of the barcode region using two short synthetic oligonucleotides, called primers, with the help of a polymerase enzyme. The PCR consists of three major steps repeated in 25 to 50 cycles. The first denaturation step serves to separate the double-strand DNA, and it is usually performed at high temperature (94 – 98° C). During the second step, the primers hybridize with the target DNA region at temperature, which depends on the primers sequences as well as the degree of specificity that is required (usually ranging between 45 and 60° C). The final step consists of the elongation of the barcode region by the polymerase, the temperature depends on the enzyme, but it is usually performed at 72° C. Each of the three steps usually lasts for 30 – 60 seconds depending on the primers or the polymerase used. As a result of the PCR reaction, the number of amplified DNA fragments, also called amplicons, is increasing exponentially, generating enough material for sequencing.

PCR primers

The choice of PCR primers is a critical step for the detection of species in eDNA samples. In the single-species detection approach, the primers need to be specific to the target species in order to avoid false positive detections. The primers must also be well validated in their application (tested against non-target species, tested on a range of environmental samples and ideally tested over temporal sampling campaigns) for the results to be interpreted by the end-user. For this approach, conventional, quantitative or digital PCR techniques are used to amplify the DNA. In the community approach, the primers need to be generic enough to amplify all species belonging to target group, but ideally not beyond that group.

The primers have to hybridize to the DNA barcoding region, which is variable between species. How primers are built, and to which extend they can be specific to a taxonomic group of interest, depends on the character of gene that shall be amplified (for an extensive overview, see Taberlet et al., 2018). There are two main types of barcoding markers: protein-coding genes (e.g., COI, rbcL) and ribosomal genes (e.g., 18S, 16S, 12S, ITS, 23S). Those two types of gene are built differently. The protein-coding genes are usually uniform through the entire gene to ensure the proper translation of the amino acids but since the genetic code is redundant, the first and third codon-base may be variable among species. This particularity is useful to ensure very good identification at the species level. However, it may be difficult to find in protein-coding genes the signatures typical for higher taxonomic rank. Therefore, the COI primers work very well for single-specimen barcoding but are much less efficient in metabarcoding. On the contrary, ribosomal genes are composed of a mosaic of conserved and variable regions. Therefore, it is easier to find genetic signatures to higher taxonomic groups in the conserved region that allows the synthesis of both highly specific primers as well as more universal primers. Usually, one or two variable regions are then used for the identification to species or genus level. Some studies use a mix of several pairs of primers targeting different variable regions in the same PCR reaction, called multiplex PCR. However, this approach may lead to some biases and decrease the efficiency of PCR reaction, if not optimally implemented. It is recommended to use one pair of primers per PCR reaction unless thorough testing for a multiplex approach has been accomplished.

PCR replicates

For each eDNA sample, several PCR reactions are usually performed, these are called PCR replicates. For one PCR reaction, only a subsample of the total extracted eDNA is used, usually 1 – 10% of the sample. A species can be missed by chance in one PCR reaction (resulting in a false negative) and PCR replicates increase the probability of finding target species' DNA present in the samples. For metabarcoding studies, two PCR replicates are seen as minimally needed to give a good overview of the targeted biodiversity. However, if the purpose is to detect a specific species (targeted approach), the number of replicates should be increased. Recommended practices for the of detection of individual species in community analysis are 7 to 15 PCR replicates for each sample. Alternatively, one can add more DNA per reaction and do less replicates.

5.4 Single-species detection

To answer certain ecological questions, the detection of a single species rather than the entire community is of interest especially in the case of non-native, invasive or illusive, and protected species. Single-species detection has been used for many studies, and covering all groups of organisms (see Table 3). Once a species-specific eDNA assay is established and rigorously tested in the lab, results can be delivered relatively fast after extraction.

Currently, in Europe there is only one species-specific eDNA assay (e.g., Thomsen et al., 2012; Rees et al., 2014b) that is already implemented on a regulatory basis (i.e., with a legal mandate behind). The eDNA detection of great crested newt (*Triturus cristatus*) has been formally recognized by the UK regulatory agency Natural England as a valid proof of physical presence of the species upon which tested habitats can be put under protection. Targeting an individual species is preferred over a whole community approach due to faster analysis time and lower costs per sample. It is important to recognize that a species-specific assay only allows the detection of the targeted species, and provides no information about the occurrence of other, even closely related, species that are not specifically looked for.

To provide the best results, all steps from eDNA sampling to extraction over to PCR should be optimized for the species of interest. The development of a species-specific PCR assay is time consuming and therefore costly. Many assays are published in the scientific literature, but the extent to which these assays have been tested in terms of specificity and sensitivity (Limits of Detection, LoD) varies greatly. Furthermore, the geographic range where the assay was validated should be considered, as the assay may not be suitable for other regions than the one it has been initially developed for. A geographic mismatch is expected when the targeted species shows local variation in the selected barcoding region or if the local species-pool differs and contains species that the assay was not tested against. In order to help practitioners to understand the developmental stage and the associated uncertainties of a selected assay, Goldberg and co-workers (2016) published critical considerations. The implementation of an assay validation scale is currently discussed (details can be found at *www.edna-validation.com/*) and can help to understand remaining uncertainties and interpretation of different developed assays.

Interpretation of single-species PCR results
It is important to recognize that molecular methods do not deliver equivalent results to traditional methods. While classical sampling often results in the collection of individuals and therefore provides information about number of individuals, eDNA results reflect the number of amplified gene copies (reads) and cannot be linked to number of individuals. Therefore, a rough guidance for interpretation of the results may be useful. It is important to acknowledge that PCR is a highly stochastic process and a positive amplification depends whether the targeted DNA molecule is present in the reaction or not. Due to the stochasticity, several PCR replicates per sample are implemented, but the number depends on the method. If all or a great majority of the replicates are positive, the detection is robust, indicating high likelihood of the species presence in the sampled environment. However, if only one or a few PCR replicates are positive, the detection is uncertain and further investigation is needed. This can be done either by increasing the number of PCR replicates or by using traditional methods. Lastly, even if all replicates end up negative and no evidence for species presence can be established, this does not implicitly mean that the target species is not present, similar to any other method resulting in negative detections too.

Currently, three different PCR approaches are used to amplify species-specific DNA enabling the detection of targeted species: conventional, quantitative and digital PCR. Conventional PCR delivers results informing about presence/absence of a target species while quantitative and digital PCR permit estimations of species abundance based on eDNA concentrations. In the following chapters, characteristics of each approach are discussed, and future opportunities are highlighted.

5.4.1 Conventional PCR

A conventional PCR assay consists of amplification using a species-specific forward and/or reverse primer. The reaction is happening in a conventional PCR machine, a heat block that is able to change between the different temperatures used in a PCR in a short time. The product of a conventional PCR is validated at the end of all cycles and is usually visualized with an agarose gel, capillary electrophoresis or on a sequencer, if fluorescent-labelled primers were used (Goldberg et al., 2011). All three options allow verification of the product according to the sequence length of an amplicon or the emitted light, but do not prove that the generated product matches the base pairs of the expected sequence.

Several studies were published based on a species-specific conventional PCR approach (e. g., Deagle et al., 2003; Jerde et al., 2011; Mahon et al., 2013; Keskin, 2014; Mächler et al., 2014; Piaggio et al., 2014). However, conventional PCR provides information on presence/absence only. Conventional PCR was for a long time popular due to lower costs per sample but with decreasing prices, it is expected that it will be rapidly replaced by other PCR approaches.

5.4.2 Quantitative PCR (qPCR)

Quantitative PCR (qPCR) offers quantification of initial DNA concentration from a sample, which is beyond possibilities of a conventional PCR. This is especially relevant, as a good number of studies found a positive relationship of the detected eDNA concentration and abundance or biomass of the targeted species (e. g., Takahara et al., 2012; Jane et al., 2015; Klymus et al., 2015). A qPCR can be performed by two different strategies, either by using a fluorescent dye or by the use of a fluorescent-labelled probe. Both strategies are based on fluorescent quantification of the PCR product and therefore need to be run in a quantitative PCR machine, which enables the detection of fluorescence.

The fluorescent dyes used in qPCR emit light with the accumulation of PCR products. Similar to a conventional PCR, an assay consists of species-specific forward and/or reverse primer and the addition of a fluorescent dye to the PCR mix. Thereby, a qPCR assay using fluorescent dye is not more specific than a conventional PCR reaction, as it is targeting all double stranded DNA. The light would be emitted regardless whether the generated product has the species-specific sequence or not; creating false positives or overestimation of the eDNA concentration. Thus, best practice would be to sequence the product as a confirmatory step.

The second strategy is to use a fluorescent-labelled probe, which is an additional nucleotide probe that binds to the target sequence during PCR and releases fluorescence upon amplification, generating a detectable signal. Similar to the primers, the designed probe must match the species-specific sequence in order to avoid non-target amplification. To design a probe-assay is more challenging, as the three elements (the probe, the forward and the reverse primers) occur together in the same assay, but must not interfere or block each other. Because quantification is based on matching these three elements, the assay is more specific and sensitive. Furthermore, multiplexing is possible with the probe strategy by using different fluorophores for different assays (up to five combinations are possible depending on the qPCR machine).

For both qPCR strategies, the quantification of DNA can be relative or absolute. A relative quantification will allow comparisons of samples analysed together in the same run. Absolute quantification of the DNA is more meaningful and often the preferred solution. To perform absolute quantification, a dilution series, based on a standard with known DNA concentration, needs to run along the samples. Rigorous testing of an assay and dilution series of the target allow a definition of the lowest amount of target DNA that can be detected (so called 'Limit of Detection', LOD) and the lowest concentration that still provides acceptable levels of precision and accuracy for quantification (so called 'Limit of Quantification' (LOQ) (Klymus et al., 2019).

Quantitative PCR approaches with a probe-based design are currently the gold standard for single-species detections (e. g., Thomsen et al., 2012; Goldberg et al., 2013; Laramie et al., 2015; Mauvisseau et al., 2018). Studies indicate that qPCR may be slightly less accurate and more expensive than digital PCR (e. g., Hunter et al., 2017) thus one can expect that qPCR approaches will be less used in the future once digital PCR will be established and more commonly available.

5.4.3 Digital PCR (dPCR)

Digital PCR is the most recent development in the PCR techniques, with different technologies and many new applications to be anticipated. The dPCR approach has already been applied in eDNA studies (Nathan et al., 2014; Doi et al., 2015; Hunter et al., 2017; Baker et al., 2018). It is similar to qPCR, as it requires a fluorescent dye or a fluorescent-labelled probe to quantify amount of DNA in a sample, but the quantification method is based on a different technique. In a digital droplet PCR (ddPCR), one of the available dPCR technologies, an individual sample is partitioned into thousands of droplets or physical partitions and each compartment either contains one or no template. Each individual compartment acts as a micro PCR reactor, where the amplification of the target sequence, if present, takes place. Then, they are analysed either by using microfluidics technology or by an optical module, determining the proportion of PCR-positive amplifications. This allows quantification of the DNA without running a standard curve along the samples and enables a more accurate quantification at low concentrations compared to qPCR. A few differently labelled assays can be multiplexed within a dPCR, similarly to the probe-based qPCR approach. So far, only one eDNA study investigated the relationships between concentration of DNA measured by dPCR and biomass and found a positive relationship (Doi et al., 2015).

5.5 Metabarcoding

5.5.1 PCR for metabarcoding

An important feature to consider when designing PCR primers for metabarcoding is the length of the amplified barcode. The barcode cannot be too short because it must be taxonomically well resolved, that is, comprise enough variations to distinguish closely related species. However, it also cannot be too long, because it otherwise does not fit technical features of sequencing technologies. Currently, most of the barcodes used in metabarcoding studies range between 200 and 500 bp. Shorter barcodes (less than 120 bp) are sometimes used, especially for macrobial species detection, but such short gene fragments are more susceptible to persist, likely more prone to transportation over long distance, and have lower taxonomic resolution.

PCR biases

Although PCR amplification is a great tool to amplify metabarcodes, it is also the main source of technical errors during the metabarcoding workflow (Berney et al., 2004; Aird et al., 2011). These technical errors include the substitutions and insertions introduced by the polymerase enzyme (Eckert & Kunkel, 1991; McInerney et al., 2014; Lee et al., 2016), the substitutions induced by the DNA damage caused by the temperature cycling of the PCR (Potapov & Ong, 2017) and the formation of chimeras (Fonseca et al., 2012). Chimeric PCR products are generated when small DNA fragments that did not finish the elongation during one step are used as "primer" in the next amplification step. The final amplicon will be a chimeric sequence that do not exist in any living organism and which is composed of two different DNA fragments that originate from two different organisms. Moreover, it is important to be aware that metabarcoding primers will not amplify all DNA equally in a sample, leading to biased abundance ratio between DNA from different species (Elbrecht & Leese, 2015; Piñol et al., 2015). All these PCR biases need to be addressed during the analysis of the sequences.

5.5.2 High-throughput sequencing

Several steps are necessary to prepare the amplicons for the sequencing. First, PCR replicates have to be pooled, purified and quantified for the library preparation. During this step, sequencing adaptors are added to each sample so that they can be demultiplexed (identified) during analysis after sequencing. Several strategies have been developed to allow the multiplexing of several samples into a single library. These include 1-step PCR, 2-step PCR or ligation-based tagging approaches (Zizka et al., 2019). The latter is by using tagged primers bearing a short series of nucleotides attached at each primer's 5'-extremity. A unique combination of these primers is used for each sample to enable the demultiplexing of the samples after sequencing (Esling et al., 2015). When multiplexing strategies are used, the PCR-free protocol for the library preparation is recommended to avoid the creation of chimeric amplicons that may originate from different samples. Finally, each library has to be verified and quantified by qPCR before running it at the sequencing machine.

The performance of different HTS platforms has been compared by several authors (Quail et al., 2012; Frey et al., 2014). Currently, the most often used sequencing technology in metabarcoding is the Illumina MiSeq. It provides the best compromise for amplicon sequencing with the sequence length up to 600 nucleotides and a maximal output of 24 million sequences. The MiSeq instrument proposes several paired-end solutions to run the samples. Four different flow cells are available to generate 1, 4, 15, or 24 million sequences respectively, with fragment length of 2 × 150, 2 × 250, and up to 2 × 300 base pairs (up to 600 with the 24M flow cell). The recommended sequencing depth for Illumina Miseq (theoretical number of sequences per sample) is commonly between 50,000 and 100,000 sequences per sample, but required sequencing depth depends on the aim of analysis and thus its effect should be analysed by using rarefaction curves.

5.5.3 Data analysis

Bioinformatic part of metabarcoding workflow comprises four main steps (Fig. 11):

1. **Quality-filtering** – Amplicon sequences with a low quality and/or ambiguous bases are removed. The

paired-end sequences are merged into a contiguous full-length sequence and potential chimeras are removed.
2. **Clustering** – High-quality sequences are clustered according to their similarity to one another and grouped into operational taxonomic units (OTUs).
3. **Taxonomic assignment** – OTUs are compared to reference database and assigned to taxa depending on their sequence similarity or other criteria.
4. **Data analysis** – The list of OTUs serves to analyse the taxonomic composition of each sample and their relation to environmental variables.

The recent developments of metabarcoding pipelines tend to overcome the clustering step by denoising HTS data and combining sequences into Amplicon Sequence Variants (ASVs) that could replace OTUs (Callahan et al., 2017).

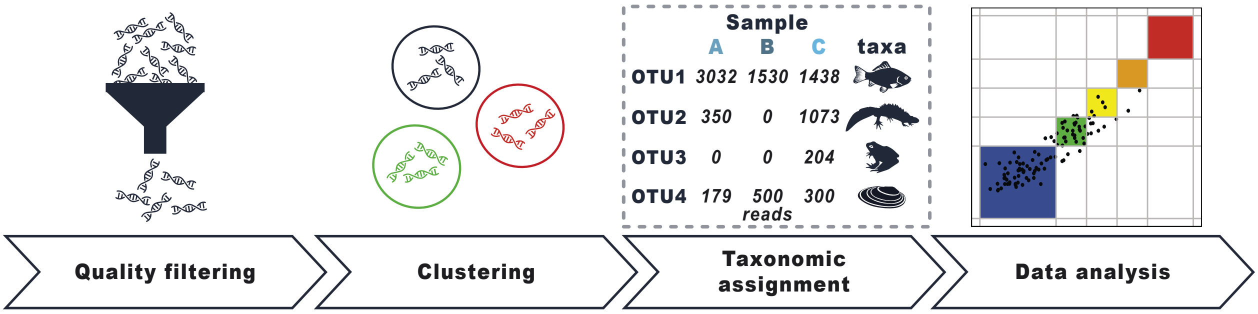
To ensure the transparency of the analysis as well as its reproducibility, it is important to document and report the bioinformatics pipeline as well as the data of intermediary steps of the cleaning process. Several algorithms are available to analyse metabarcoding data. Most of them are in command-line interface (QIIME, MOTHUR, DADA2, Obitools), but some of them are proposed with user-friendly graphical interface (SLIM, Dufresne et al., 2019). Since bioinformatics is a very fast evolving discipline with new algorithms emerging constantly, there is relatively little consensus in the scientific community about which algorithms to use.

Interpretation of metabarcoding results

It is important to always use the same pipeline when comparing metabarcoding data, keeping in mind that it is possible to go back to the original sequence file to run it through a new pipeline, if necessary. The degree of expertise of the person who interpret metabarcoding data is highly valuable and several issues are important to keep in mind. The data generated are usually presented as a matrix with the list of species (or OTUs) and the number of sequences found in each sample for each species. This number can be highly variable, and it represents a major issue in term of quantitative interpretation of metabarcoding data. For abundant taxa, several studies showed a correlation between the relative numbers of sequences and the relative abundance of specimens within a taxonomic group (Evans et al., 2016; Hänfling et al., 2016, Schenk et al., 2019). Although promising, those conclusions are very specific and cannot be generalised to all metabarcoding studies. The interpretation may be even more problematic with rare species. Since metabarcoding has been shown to be highly sensitive, the presence of some species could be easily overestimated. Even with a meticulous care and appropriate materials and equipment during the sampling and the laboratory work, it is impossible to get rid of all cross-contaminations, including tag jumping. The common answer to this issue is the application of a threshold on the number of sequences to avoid the false positive. However, there is no consensus on the threshold that should be applied. Usually thresholds are species specific and based on the analysis of a lot of controlled samples (Harper et al., 2018). The use of positive and negative controls during sequencing could help resolving this issue.

Figure 11

Workflow of high-throughput sequence data analysis



6 Reference database for taxonomic assignment

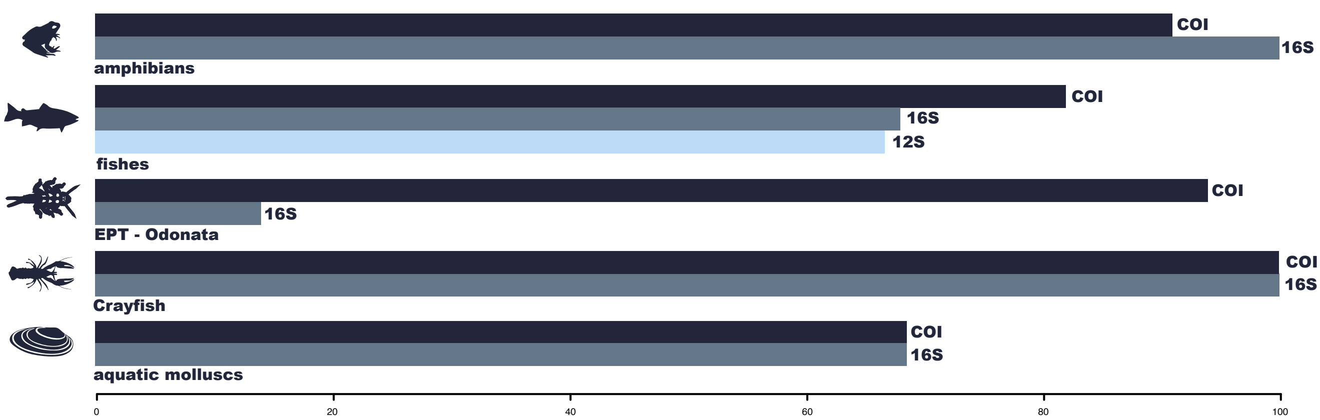
Taxonomic assignment is a crucial step in metabarcoding study as it allows to relate the DNA sequences to morphospecies. To do so, one needs a high-quality curated reference database. Incomplete reference databases are the major factor limiting the assignment of sequences to taxonomic names. Even for common bioindicator taxa there are still important gaps (Weigand et al., 2019). However, several international and national initiatives are committed to fill these gaps. First, at national level the Swiss Global Biodiversity Information Facility (GBIF.ch) is centralizing DNA sequences linked to species observations or specimens in museum collections. These data are processed in order to build a Swiss reference database that comprises all the genetic data for all species present in Switzerland. The GBIF.ch database will be publicly available in 2021 with mention of different quality levels associated to each sequence. The different quality levels depend on the quality of the DNA data itself (Sanger sequencing source file), but also on the traceability of the genetic material at the origin of the DNA sequences and the reliability of the species determination. Documentation of the DNA and/or tissue sample will be available, as well as a ranking for genetic sequences according to the type of voucher (holotype, paratype, topotype, vouchered specimen, or its photo). Most of the data available for now concern terrestrial species but the number of records for aquatic species is rapidly growing (for example all Plecoptera and Trichoptera species living in Switzerland have just been sequenced and the database for Oligochaete species is well advanced). The database coverage of major aquatic taxa in Switzerland is illustrated in Figure 12.

In addition to this local database, several international online databases can also be used for taxonomic assignment. The most common are BOLD (*http://v4.boldsystems.org/*) and MIDORI (*http://reference-midori.info/server.php*) for COI marker, SILVA (*www.arb-silva.de/*) for ribosomal markers, Diat.barcode specifically for diatoms rbcL markers (Rimet et al., 2019) and GenBank (*www.ncbi.nlm.nih.gov/genbank/*) for all markers. However, it is important to be careful about the quality of the sequence and assignment, especially with the databases that are not curated (GenBank, MIDORI). It may be useful to build a local reference database with the taxonomic groups of interest and curate it locally.

Figure 12

Database coverage (in %) for species of specific aquatic taxa present in Switzerland

The coverage is given for the COI and the 16S barcode region for all groups and 12S for fish. EPT refers to Ephemeroptera, Plecoptera and Trichoptera



7 Data management

Every eDNA study must follow a data management plan similar to other genetic studies. That means that all relevant information about eDNA data generation, analysis and storage is documented and made available. It is important to clearly define the requirements for data management when planning an eDNA study. In particular, it is recommended to consider following issues:

- Clarify, what happens to the eDNA extracts.
- Plan and document the long-term storage of the DNA extracts, preferentially with biobanking networks.
- Make all laboratory protocols (type of DNA extraction, primers used, PCR settings, sequencing) available.
- Make the parameters of sequence data filtering and further bioinformatics analysis available.
- Define and provide the reference database for taxonomic assignments; ideally, a public reference database.
- Define where and how the raw sequence data and the final processed sequence dataset is stored and available.
- Define ownership of the data. Best practices are to submit all generated sequences to a public database (e. g., European Nucleotide Archive).

The data management plan ensures that the eDNA study will be conducted in agreement with best practices and standards commonly accepted in the field. There is substantial variation in the lab techniques used for eDNA extraction and processing. Therefore, it is recommended that the general methodology is described in the report and more detailed information is available upon request. This is essential, if the particular eDNA study is to be repeated or compared to other similar study.

In Switzerland, a matrix to submit species observations issued from eDNA projects has been developed by national institutions (GBIF.ch, *www.gbif.ch*; InfoSpecies, *www.infospecies.ch*; SwissBOL, *www.swissbol.ch*) and is recommended to be used. This matrix follows InfoSpecies basic requirements and comprises information on the molecular workflow, so that species observations based on eDNA data can be validated by InfoSpecies. This also comprises information on the nature of sample, the people involved in the process as well as the database used for the taxonomic assignment and the reliability of the results (for example, number of sample replicates that gave positive qPCR results for a given species). Linking the report of the project in pdf format is highly recommended.

8 Application examples (case studies)

8.1 Single-species detection

Species-specific assays can be generated for any desired species, but the development of an assay is simpler if DNA barcodes for a given species are already available. In scientific literature, numerous assays for a broad range of species have been established and selection is presented in Table 3. The following two case studies are examples of specific relevance for Switzerland.

8.1.1 Quagga mussel

The quagga mussel (*Dreissena rostriformis bugensis*) is a recent invader into European freshwater systems. It originates from the Ponto-Caspian region and has migrated through the Main-Danube canal into the river Rhine system, similar to its closely relative, the zebra mussel (*Dreissena polymorpha*). Unlike its sister species though, the quagga mussel can persist in low nutrient conditions and cold habitats and therefore is able to colonize deeper zones. This can cause problems for drinking water providers, especially clogging of water pipes. Invasive mussels can be discovered through their veliger larvae by scanning a water samples with a microscope.

In Switzerland, the distribution of the two invasive *Dreissena* species in the river Rhine catchment was studied using eDNA by De Ventura et al. (2017). At the time of the study, the zebra mussel was already present in the whole river system while the quagga mussel was found up to Kehl (Germany), but not present in Switzerland. De Ventura and colleagues discovered for the first time quagga mussel eDNA at Basel (Switzerland) and informed about the presence of the invasive species before any other observation method did. The result created great interest of the federal government and cantonal agencies, raised awareness and intensified monitoring of this invasive species. In May 2016, divers observed for the first time the quagga mussel in Lake Constance at a depth of 25 m. Currently the species is present everywhere in Lake Constance as well as in Lake Geneva and possibly in other large lakes in Switzerland.

8.1.2 Round goby

The round goby (*Neogobius melanostomus*) is one of five goby species that are expanding their territories into the Rhine system. From the Ponto-Caspian region, where the round goby is originating from, the species likely dispersed as larvae in the ballast water of ships into the Rhine. The round goby threatens native species due to competition for food and nesting sites, shows aggressive behaviour, and eats eggs of native fish species. In 2012, the round goby was discovered for the first time in the Higher Rhine (Kalchhauser et al., 2013). The Federal Office for the Environment put the round goby on the list of invasive species in 2017. Traditionally, fishes are surveyed through electrofishing, but species as the round goby are hard to detect with this method, because they hide between stones in the interstitial of large rivers.

In 2016, the feasibility of using eDNA to detect *N. melanostomus* was tested in the river Rhine (Adrian-Kalchhauser & Burkhardt-Holm, 2016). In an extensive study, best practices for eDNA detection of round goby were developed, and a new invasion front of the species was detected. Sampling at the river bottom, close to the species habitat, resulted in better detections compared to samples from the surface water. The testing of different laboratory protocols showed that certain extraction methods or inhibition could prevent successful detections of the round goby. Such species-specific adaptations of sampling and laboratory methods are required in order to establish a sensitive assay.

8.2 Vertebrates diversity (example newts)

Five species and one subspecies of newts occur in Switzerland: *Lissotriton helveticus*, *Lissotriton vulgaris*, *Lissotriton vulgaris meridionalis*, *Ichthyosaura alpestris*, *Triturus cristatus*, and *Triturus carnifex*. Their natural distribution differs between regions. Some of them (e. g., *Lissotriton vulgaris meridionalis*) are considered as native in some cantons (Ticino) and treated as non-native species in others (Geneva). Therefore, it is important to develop genetic tools that allows to survey the whole diversity of newts, rather than detecting a single species (Harper et al., 2018).

In 2018 – 2019, a study conducted at the University of Geneva (Charvoz et al., 2019) developed an eDNA metabarcoding test that was used to monitor the diversity of newts in Geneva region. The test is based on a mitochondrial 16S barcode that allows distinction of all species and subspecies of newts present in Switzerland, except the hybrids of *T. cristatus / T. carnifex*. The specificity of the selected barcode averages 65% of sequences that could be assigned to newts. The remaining sequences were assigned to fish and birds. During the study, the presence of newts was checked in 30 ponds by visual observation and water eDNA analysis. The newts' DNA was detected in all sites where the newts were observed with perfect correspondence between sequenced and observed species. Moreover, the newts' DNA were found in several ponds where the species was not observed. This was particularly striking in the case of the invasive species *Lissotriton vulgaris meridionalis*, which DNA was found in 7 out of 19 ponds where no individuals were observed. For further studies on detection of newts by eDNA in Switzerland, see Dubey et al. (2019) and Cruickshank et al. (2019).

8.3 Macroinvertebrates

Macroinvertebrates reflect a broad spectrum of diversity and belong to one of the main bioindicator groups (beside diatoms and fish) regularly investigated for water quality assessments. Thus, a great interest persists to apply molecular tools for the identification of macroinvertebrates communities. A major challenge is that "macroinvertebrates" are a phylogenetically highly diverse and polyphyletic group, such that many non-target taxa not considered as macroinvertebrates, such as rotifers, are also amplified and sequenced (Deiner et al, 2016). Importantly, these latter organisms have not been used in classic assessments (e.g., Stucki 2010) because they are not accessible using classic methods or there is a lack of knowledge to morphologically identify them. Thus, metabarcoding approaches based on DNA extracted from tissues (bulk samples) might be more comparable to current, classical sampling methods and implementation might be straightforward in a short term (Blackman et al., 2019). Global approaches based on water DNA metabarcoding might be rather useful to expand the taxa covered (e.g., to include meiofauna-sized invertebrates).

8.3.1 Water eDNA (global approach)

A large comparison of macroinvertebrate assessments (kick-net sampling) and eDNA metabarcoding has been conducted by Mächler et al. (2019). They collected water DNA and kick-net samples at 61 sites distributed over a large river network (Thur catchment, 700 km^2), with a focus on the genus level within Ephemeroptera, Plecoptera and Trichoptera (EPT), using the COI barcode region. At the catchment scale (gamma diversity), both approaches detected similar proportions of the overall and cumulative richness at genus level, namely 42 % and 46 %, respectively. There was also a good overlap (62 %) between genera found in the eDNA and kick-net samples at the regional scale. Furthermore, also at the site scale the observed local taxon richness (alpha diversity) between eDNA and kick-net samples was highly congruent. Local richness of macroinvertebrates found in eDNA samples was positively related to discharge (see also Deiner et al., 2016), and the identity overlap (i.e., identity of genera) found by both methods at the local scale was less good. This indicates that transportation processes of eDNA do affect the local sample, such that the taxa found are a mix of locally occurring taxa as well as further upstream signals.

Similarly, Fernández et al. (2018) did a study on macroinvertebrates, comparing traditional monitoring with eDNA sampling at six sites of the river Nalón (Spain). They tested three different barcodes: two based on the 18S and one on the COI region. The overlap with traditional methods was higher with the COI barcode (56.3%), than with 18S (20.6%). However, some families (Chloroperlidae, Elmidae, Lumbricidae, Phylopotamidae, and Sphaeriidae) remained undetected by all three barcodes. The results revealed that the different barcoding regions target different taxonomic groups. Most relevant detections with the COI where on the following phyla in the order of decreased detections: Arthropoda > Cnidaria > Annelida > Mollusca, wereas phyla and their order was different for the 18S (Nematoda > Porifera > Arthropoda > Cnidaria). Overall, more families were detected with the COI primer than with traditional sampling, and 18S primers detected the lowest number of families.

8.3.2 Bulk DNA (kick-net samples)

Elbrecht and colleagues (2017) performed a study comparing ecological quality ratio (EQR) of 18 Finish riverine sites based on morphological and molecular identification. To do so, the scientists collected benthic macroinvertebrates following national guidelines for monitoring with a kick-net and preserved specimens in the field. Morphological analyses were performed by experts as part of the routine national monitoring program and specimens were identified to species or genus level, except for Oligochaeta, Turbellaria, Nematoda, Hydrozoa, and the two dipteran families of Chironomidae and Simuliidae. After morphological identification, the specimens were dried, homogenized and used for DNA extraction. Metabarcoding of the tissue-derived DNA was done with universal COI primers adjusted for macroinvertebrates (Elbrecht & Leese, 2017). The results of morphological- and DNA-based assessments were significantly correlated, but the final category derived by the two assessments occasionally differed by one (e. g., "Good" instead of "Moderate"). The metabarcoding approach detected more than double the number of taxa and improved taxonomic resolution where morphological identification was restricted to family level (Limnephilidae) or genus level (Eloeophila, Hydroptila, Baetis-complex). However, about $32.5 \pm 9.7\%$ taxa per sample were not identified with the metabarcoding approach due to several reasons such as primer bias, incomplete reference database or poor DNA preservation. The authors of the study suggest using of molecular grade ethanol to conserve specimens in the field and improve quality of bulk DNA.

8.4 Biotic indices

8.4.1 Swiss Molecular Diatom Index (MDI-CH)

Current legislation recommends using benthic diatoms to assess the ecological status of rivers and streams. Diatoms have been chosen because they are highly sensitive to environmental conditions and respond quickly to changes in physicochemical and biological factors. In different countries various biotic indices have been developed to assess environmental impact using diatoms. Most of these indices are based on the relative frequency of species weighted by their autecological value. In Switzerland, the Swiss Diatom Index (DI-CH) was proposed in order to characterize the biological status of rivers and streams using the frequencies and distributions of more than 400 diatom species and morphological varieties (Hürlimann & Niederhauser, 2007). The DI-CH is based on chemical parameters indicating anthropogenic pollution and classifies the water quality into 5 different ecological classes on a scale from 1 to 8. The calculation follows the weighted average equation, which involves an autecological value D and a weighting factor G, which are specific to each species. It also uses an additional parameter H, which corresponds to the relative frequency of a particular taxon in the sample. Traditionally, the Swiss diatom index (DI-CH), as well as the diatom indices in other countries, is calculated based on microscopic analysis of diatoms community. The diatoms are isolated from biofilm samples and the diatom frustules are identified following the guidelines (Hürlimann & Niederhauser, 2007), which contain also the ecological values and weighting factors assigned to each morphospecies.

A molecular index could present several advantages for routine assessment. First the quality assessment will be comparable to the morphological index therefore can easily be used to complement of the traditional method. Moreover, since a lot of samples can be processed in the same time in the lab, a molecular index could be very useful for large-scale survey. Since 2014, several studies have been conducted in Europe to test the application of eDNA metabarcoding to assess the composition of diatoms community and calculate water quality indices (Kermarrec et al., 2014; Zimmerman et al., 2015; Vasselon et al., 2017a; Keck et al., 2018). The purposes of these studies were to test different markers, complete the reference database and analyse the correlation between morphological and molecular data, in term of species composition and/or value of water quality index. In Switzerland, the Swiss Molecular Diatom Index (MDI-CH) is currently under development and provides very promising results (Visco et al., 2015; Apothéloz-Perret-Gentil et al., 2017). The on-going studies extend the geographic range of sampling localities by analysing diatoms data from NAWA SPEZ and NAWA TREND campaigns (BAFU, 2013), also through the EU Interreg Program SYNAQUA (Lefrançois et al., 2017) and in collaboration with Swiss cantons, environmental consultancies and French researchers from National Institute of Agricultural Research (INRA).

8.4.2 Genetic Oligochaete Index of Sediment Bioindication

Sediments are an essential component of river and lake ecosystems and they also have the property of storing certain types of contaminants. Some pollutants can reach concentrations sufficient to induce adverse effects on benthic organisms and thus disrupt the proper functioning of the ecosystem. Oligochaetes are good bioindicators of sediment quality as they are restricted to this compartment, display low mobility, and their trophic mode is primarily collector based on the ingestion of fine sediments. In addition, the group includes a large number of species presenting a wide range of pollution sensitivity (Rodriguez & Reynoldson, 2011) and oligochaetes are generally abundant in sediments (Vivien et al., 2014). Different biological indices based on the study of the structure of oligochaete communities have been developed for the assessment of the biological quality of stream and lake sediments. Among them, the Oligochaetes Index of Sediment Bioindication (IOBS) allows to assess the biological quality of fine/sandy sediments in streams (AFNOR T90-393 2016) and the Oligochaete Index of Lake Bioindication (IOBL index) to describe both the state of functioning and the biological quality of sediments (AFNOR T90-393 2016). The IOBS index has been applied in Switzerland for ten years as part of programs of quality monitoring of stream quality and on an ad hoc basis (Vivien et al., 2014, 2015a). In addition, oligochaete communities have regularly been studied in Switzerland for several decades to assess the biological quality of lake sediments. However, the implementation of oligochaete indices requires solid expertise in oligochaete taxonomy. In this context, the development of an index based on the identification of oligochaetes using genetic barcodes would allow to solve the problems associated with the identification of species and a wider use of oligochaetes as bioindicators.

Since 2013, a project aiming at the development of oligochaete genetic indices is conducted in Switzerland. The project led to the creation of reference database of aquatic oligochaetes DNA barcodes, based on the analysis of specimens collected in Switzerland (Vivien et al., 2015b). In parallel to the development of reference databases, the DNA metabarcoding approaches were applied to assess sediment quality using oligochaetes. The preliminary studies show that, despite significant differences between the morphological and molecular approaches concerning the presence/absence and the abundances of taxa, the development of such approaches was possible by adapting the index calculation and delimitation of quality classes (Vivien et al., 2016, 2019). Another method based on high-throughput barcoding of oligochaetes was validated as part of the INTERREG SYNAQUA project (Lefrançois et al., 2017). It presents the advantage of reliably and accurately estimating species abundances at a site. At all sites tested (stream and lake), the diagnoses of biological quality established with the morphological and molecular approaches were concordant (Vivien et al., 2019).

9 Conclusions and outlook

The detection of DNA from specific species in environmental samples and scalability of metabarcoding-based technologies are fundamentally transforming the way biodiversity is monitored and how bioassessment is done. The detection technology and techniques summarized and described in this publication offer some major advantages compared to classic approaches. The most important advantage is the ability to identify and monitor a very broad range of organisms, virtually ranging from microbes to plants and animals, with an eDNA-based approach. This not only allows the integration and better resolution of "classic" bioindicator groups used in biodiversity monitoring or bioassessment, but also opens up opportunities to use hitherto underused or even ignored groups for the same purpose. Highly diverse groups, such as Diptera or Oligochaetes that are challenging to identify to the species level, are largely not considered in classic approaches, even though they may convey complementary and highly valuable information on the state of biodiversity and the ecosystem. eDNA-based technologies can allow to fully exploit the information provided by these groups. Further, the technologies described here have the potential, some already implemented, to be conducted in (semi)automated manner. The use of laboratory-robots, the rapid advances of sequencing technologies and the ability to link the eDNA sampling to regular water sampling for chemical analyses has the potential to provide more data, at a faster rate, and a cheaper price.

A further fundamental advantage is that for all of the herein described eDNA approaches, the sampling is non-invasive for large sized, macrobial organisms, whose presence is inferred from DNA traces they leave in the environment. The ability to detect organisms in the environment without collecting or harming these organisms is especially important for rare or endangered species and where such sampling is ethically problematic or restricted. This is principally the case for fish, amphibians and other vertebrates where classic sampling often involves direct manipulation or even killing of the specimens, which is generally not wanted. Gaining the same or similar information on the occurrence of these species based on an environmental sample is highly advantageous.

Finally, the use of environmental DNA samples to assess biodiversity or for bioindication allows a partition of work: the sampling in the field is less dependent on specific technologies or infrastructure and people can be trained relatively easily. Thus, field sampling can be done by stakeholders themselves and eDNA samples can be stored and transported to specialised molecular laboratories. These laboratories can then centrally process large number of samples under highly standardized conditions. This, and the generally more rapid sampling process itself, may allow that more (both spatially and temporally) samples can be taken. A spatially higher sampling coverage will improve the resolution of biodiversity data and lead to more effective management.

However, while the "new generation" of biomonitoring and bioassessment using eDNA-based tools currently opens up many opportunities, it is not without its challenges. For example, the eDNA approaches presented here are based on novel biotechnologies that undergo rapid modifications and improvements. Thus, in a period of development and transition, as currently faced, it is not easy to define and set long-lasting standards, and methodological changes are expected to affect some recommendations defined in this report.

Some challenges of the eDNA approaches are not likely to be overcome, because of the specific character of eDNA. For example, eDNA does not provide information about the demographic structure, the age of individuals or health status, or whether the organisms were alive or dead at the moment of sampling. In addition, the actual number of organisms (i. e., abundance) is difficult to infer from metabarcoding data due to various biological and technical factors. While the relation between number of sequences and biomass or abundance could be established for some fish species, there are no universal analytic tools that would provide the reliable establishment of quantitative abundance data yet. However, especially for single-species approaches, the latter might be resolvable.

Data generated by eDNA approaches may differ in quality and quantity from existing classic approaches, such that they will give different types of information, not necessarily better or worse than classic approaches, but often complementary to them. It is important to not constrain the novel approaches by propagating limitations of classical methodology. For example, while metabarcoding of bulk samples will give diversity data more directly comparable to classic kick-net sampling, it cannot be speed up beyond the limitations of the kick-net sampling and specimen sorting steps needed to obtain the bulk sample. With data derived from eDNA, the individual data points are associated with a higher uncertainty and shall be interpreted in a more probabilistic than deterministic perspective. Analysis and interpretation of these data may require different statistical tools, such as Bayesian statistics, and a better understanding of false positive versus false negative records, or the origin of these errors. Much research in this direction is underway, while the quantification of uncertainty is generally not available for past observation-based techniques. Furthermore, the scale and inference in terms of space and time are potentially different. eDNA rather describes regional (watershed) than local properties and depending on the sampled environment, it could be sampled at a much higher temporal resolution.

Finally, the use of eDNA technologies is not to replace the classical approaches but to use their full potential to ameliorate and expand biomonitoring in general. For example, high throughput metabarcoding generates huge amounts of sequence data that cannot be assigned to any know taxa, but can comprise important ecological information. Today, with the help of artificial intelligence tools such as supervised machine learning, it is possible to use these unassigned sequence datasets through comparison with reference database containing millions of sequences assigned to known ecological status. The immense advantage of this type of analyses is that they not only consider a species' presence/absence or relative abundance but also include network analyses that provide information about the relations between different species and their response to environmental variables.

Overall, the advantages are convincing and the novel opportunities outweigh the challenges, making us confident that the future biomonitoring or bioassessment will include environmental DNA approaches. We hope that this publication will facilitate the comprehension of eDNA-based methods and will contribute to their propagation and implementation.

10 Sampling protocols

10.1 Water eDNA

This protocol has been developed for the eDNA sampling campaign of NAWA Trend 2018/19 for small creeks and rivers. It should be regarded as a guideline for filtration of water eDNA samples by hand and is based on best knowledge but also balancing practical aspects. Generally, clean working procedures need to be followed in order to minimize cross-contamination. Additionally, continuous cooling of eDNA samples is needed for the successful outcome in this protocol.

Note: This protocol can be easily modified to perform filtration using a vacuum pump. This is especially suitable if larger volumes of water or large number of samples should be processed.

Material

Per sampling site:

- 2 × sterile 50 mL single-use syringe
- 4 × Sterivex filters
- 8 × Luer-Lock caps
- 2 × Ziplock bags

Additional field material:

- 1 L deionized or distilled water and sterile scalpel for negative controls
- Waterproof pen for labelling
- Cooling box with cooling elements to store eDNA samples during field work
- Gloves
- Waste bag

Field sampling protocol

1. Put gloves on.

2. Take new syringe out of wrapping.

3. Take Sterivex filter out of wrapping.


4. Take sample from the waterside. Avoid stepping into the waterbody to prevent cross-contamination and stirring up sediments. Select a representative site and sample the water about 30 cm off the bank. Sample water about 5 cm below surface.

5. Fill syringe directly with water from the river, take exactly 50 mL without any air bubbles. In case there are bubbles, hold the syringe upright and push them out.


6. Screw Sterivex filter onto full syringe.

7. Push water at regular speed through filter. Best to hold onto filter in order not to lose the filter in case it falls off.

8. Unscrew syringe from Sterivex filter.

9. Repeat steps 5 – 8 ten times, in order to filter 500 mL water through the filter. Due to sediment particles, it may not always be possible to filter 500 mL on a single filter. In this case, it is important to note the filtered volume.

10. Unscrew syringe from Sterivex filter and fill syringe with air. Screw syringe onto Sterivex filter.

11. Push air at equal path through filter in order to get rid of water in the filter housing.

Before pushing air through: After pushing air through:

12. Put syringe back into wrapping.

13. Close filter housing with Luer-Lock caps on both sides.

14. Label closed Sterivex filter with a unique identifier.

15. Put filter housing into ziplock bag and label bag with sampling and bank side.

16. Put ziplock bag in the cooling box.

17. Note the filtered volume.

18. Take second Sterivex filter and repeat steps 4 – 14 with the same syringe.

19. Put second Sterivex into the ziplock containing the first Sterivex filter. Close ziplock properly and put in cooling box.

20. Repeat steps 2 – 19 on the other bank side of the river. eDNA must be sampled above the waded through site in order to reduce contaminations.

21. Note filtered volume and discard gloves.

22. After fieldwork the bags containing the filters need to be stored at – 20° C until extraction.

Field sampling of eDNA for negative control (also referred to as "field equipment blank")

Follow the same workflow as for eDNA samples described above. Instead of river water, however, deionized or distilled-like water is used for filtration and a new pair of gloves. The negative control should only be opened at the field-sampling site. The container can be opened with a sterile scalpel in case the syringe does not fit into the mouth of the container.

10.2 Sediment eDNA

Material (single use consumables)

- Gloves
- 50 mL syringe
- Plastic cup
- Plastic stirrer
- 50 mL tube

Note: The end of the syringe must be cut before sampling. A sharp, bleach-cleaned knife or saw may be used. Please decontaminate the cut syringe with one-hour exposition to UV light before sampling.

Note: This protocol is made for fine sediment. For coarse sediment, it may not work well. In that case, scrap the surface of the sediment with a sterile spoon and put it in the plastic cup. As for coring, repeat the sampling 3 times.

Protocol

1. After removing the plunger, push the syringe into the sediment, up to the 50 mL mark.

2. Place the plunger back on the syringe and push the rest of the air out. Then remove the syringe from the sediment. The sediment core must hold into the syringe, but be careful during this step because coarser sediment can fall.

3. It might be important to remove the surface sediment to avoid collecting phytobenthos. In this case push the sediment to the 20 mL mark.

4. Push 10 mL of sediment into the plastic cup and rinse the syringe into the river.

5. Repeat the step 1 to 4 three times at different point in the river.
6. Mix all the sediment collected with the plastic stirrer and pour the sediment into the 50 mL tube.

7. Label the tube and freeze it at −20 °C. Indicate if coring or scraping method was used for sampling.

10.3 Biofilm eDNA

Material (single use consumables)
- Toothbrushes
- Plastic plate
- Sterile pipets
- 3 × 2 mL tubes with DNA preservation buffer
- Ziplock bag

Note: The sampling procedure is based on the diatom module published by the Swiss Federal Office for the Environment (Hürlimann & Niederhauser, 2007). Requirements requested for morphological sampling are also mandatory for molecular application.

Note: For each sampling site, three samples are taken (replicates) and transferred to 3 sampling tubes.

Note: Sampling material should be discarded after use. A new set of sampling material is used for each sampling site. The same stones can be used for both morphological and molecular analysis.

Protocol
1. Wet the toothbrush in river water
2. Collect 3 to 5 stones according to the sampling procedure of the diatom module (Hürlimann & Niederhauser 2007) using single use equipment.

3. Scrape the surface of the stones over the plastic plate with help of a single-use toothbrush.

4. Collect about 1 mL of the biofilm with the pipet and transfer it into a provided tube containing preservation buffer. Repeat this step 3 times for each sampling site.

5. Label the tube and fill in sampling details in the list.
6. Put the tubes in the provided ziplock bag and preserve them at – 20° C.

11 Best practices and documentation of procedures for eDNA approaches

The provided bullet-point checklist is listing the major relevant aspects to be covered for a replicable use of eDNA for single-species detection and metabarcoding by stakeholders. These points are generally accepted norms to be minimally fulfilled for comparable use and application of eDNA approaches. The checklist shall specifically help practitioners/ stakeholders to identify the important aspects that need to be considered for eDNA based biodiversity monitoring and bioassessment.

Sampling

The following points are important guidelines to be followed by anyone conducting the eDNA sampling, which are very likely the practitioners themselves.

- People taking eDNA samples must have received a special training.
- Sampling method (e. g., precipitation, filtration, volume sampled, bulk sampling) and all sampling material (filter type, etc.) must be well documented.
- The use of disposable consumables (e. g., gloves, single-use filters, single-use toothbrushes for diatom collection) must be clearly indicated and differentiated from multi-use material.
- Cleaning procedures for multi-use field equipment is documented and clarified. Cleaning procedures must remove/degrade DNA. The use of ethanol is not sufficient. Items should be fully covered with bleach (sodium hypochlorite; NaOCl) at a minimum concentration of 1.5% NaOCl for a minimum of 1 minute, then rinsed with DNA-free water to remove all traces of bleach. UV light treatment, "DNA away" or similar products can also be used.
- Detailed sampling site description, including geographic coordinates and water depth if relevant, must be provided.
- Appropriate negative controls must be taken in the field and analysed throughout the whole laboratory workflow.
- Preservation of samples (constant freezing-chain or use of appropriate buffer solutions) must be ensured and documented.

Laboratory setting

The setting of laboratories performing eDNA based work should be subject of certification. The molecular laboratory must respect the norm of ISO 17025 or equivalent. In addition, following points should be addressed specifically in the case of eDNA analysis.

- The laboratory in which eDNA is extracted and further analysed must be organised in agreement with best practices to ensure high quality of eDNA analyses. In particular, it must possess special rooms and equipment dedicated to eDNA extraction, pre-PCR and post-PCR steps, as indicated in chapter 5.
- Post-PCR products and equipment used for handling post-PCR products (e. g., pipettes) shall never enter the clean lab. The workflow of products and personnel must be unidirectional.
- The clean lab should ideally have a positive air pressure to repel contamination. The air entering the clean lab through ventilation systems must be filtered to avoid contamination (e. g., HEPA filter).
- The clean lab must have walls and furniture that are easy to clean (e. g., with bleach or other DNA-degradable substances; ethanol is not sufficient) and cleaning should be regularly implemented.
- Laboratory personnel working in the clean lab must wear protective clothes to avoid contamination of the samples.
- All work is done preferably under hoods to protect the samples from contaminations.
- For the preparation of DNA-free reagents such as aliquots of primers and other chemicals, separate and clearly designated laboratory equipment (pipettes, sterile bench, etc.) and space is used.

Molecular laboratory work

As the field of eDNA and associated technologies is rapidly evolving, accurate reporting is essential and important points are mentioned in the list.

- Detailed laboratory protocols used at each step, from eDNA extraction to sequencing, including PCR primers and PCR conditions, must be provided or referenced.
- The negative and positive controls must be included and their results provided.
- Level of validation (*www.edna-validation.com*) for species-specific assays need to be provided.
- The number of technical replicates and their processing must be indicated.
- The characteristics of sequencing platform and the specific settings for the sequencing runs must be indicated.
- The long-term storage of eDNA extracts and other products resulting from molecular analyses should be a subject of specific agreement.

Data processing and storage

Any aspects concerning data processing and storage must be discussed between customers and subcontractors and be clearly defined in the contract.

- The subcontractor can be asked to provide or make available in open databases:
  - The complete raw sequence data (unmodified output from sequencer)
  - The filtered sequence data (after bioinformatic processing)
  - The OTUs/ASVs table with relative frequencies (after taxonomic assignment)
- The report of sequence data analyses must include:
  - A reference or documentation to the bioinformatic pipeline used for sequence data analysis, and specifically, parameters and thresholds used for sequence data filtering
  - A reference to the database used for taxonomic assignment
  - A documentation of statistical analyses and the interpretation of their results

Glossary

Adapter
Short nucleotide sequences attached to DNA sequences through HTS workflow. Adapters are used to back track DNA sequences to the original sample during bioinformatic analysis.

Amplicon
A fragment of DNA amplified through PCR.

Amplicon sequence variants (ASVs)
Individual DNA sequences produced by high-throughput amplicon sequencing after the removal of spurious sequences generated during PCR amplification and sequencing.

Bulk sample
A sample consisting of whole organisms and their fragments originating from the environment and collected manually (e. g., using kick-net sampling).

Chimeras
Genomic artefacts created during PCR amplification by combining DNA fragments of different origins.

Clustering
The assembling of similar sequences based on a fixed similarity threshold or other method leading to formation of OTUs (see below).

Contamination
Presence of extraneous DNA, which do not originate from the sample.

Cryptic species
Species that cannot be distinguished by their morphological features.

Digital PCR (dPCR)
A PCR technique in which a sample is partitioned into thousands of subsamples (droplets in digital droplet PCR). A PCR reaction occurs within each subsample and successful amplification is detected by fluorescence.

DNA barcodes
Short DNA fragments of a genetic marker allowing species identification.

DNA extraction
A laboratory process of chemical and physical steps to release and purify DNA from cells or other material.

DNA precipitation
A technique to concentrate DNA from an aqueous solution through adding salt and ethanol to the solution leading to the settlement (i.e., precipitation) of DNA.

Environmental DNA (eDNA, *sensu lato*)
Pool of genomic material originated from living organisms or their traces (such as skin cells, mucus, scales, urine, faeces, saliva, gametes, or deceased remains) present in various environments, such as water, sediment, soil, or air.

Genetic marker
A genomic DNA region (e. g., fragment of COI gene, or V9 region of 18S rRNA gene), which allows to identify species within a particular taxonomic group.

High-throughput sequencing (HTS)
A method producing millions of DNA sequences through massively parallel sequencing technologies, also known as next-generation sequencing (NGS).

Library
A collection of DNA fragments prepared for high-throughput sequencing. Each DNA fragment is flanked with specific adapters to both ends.

Metabarcode
The sequences resulting from metabarcoding and produced by high-throughput amplicon sequencing.

Metabarcoding
An approach to identify multiple species in a complex sample (e. g., eDNA or bulk sample) based on high-throughput amplicon sequencing.

Mitochondria
An organelle found in most eukaryotic organisms used in energy pathways in the cells. Mitochondrial genes evolve more rapidly than nuclear genome and therefore are often used as DNA barcodes (e.g., COI, 16S, or 12S).

Multiplexing
An approach consisting in simultaneous PCR amplification of different markers or sequencing of different samples in order to optimize the molecular workflow.

Negative controls
Measures that allow tracking potential contaminations during field sampling, DNA extraction, and PCR.

Operational taxonomic unit (OTU)
A cluster of sequences grouped by similarity that are considered as a proxy for molecular species.

PCR inhibitor
A factor preventing or limiting amplification of DNA during PCR through interaction with the DNA template, polymerase enzyme or other cofactors used in the PCR.

Polymerase
An enzyme that synthesizes DNA molecules during PCR by replicating an existing DNA sequence.

Polymerase chain reaction (PCR)
A process to generate copies of a particular fragment of DNA with the help of DNA polymerase.

Preservative solution
A compound added to samples for long-term storage (e.g., ethanol).

Primer
A short single-stranded piece of DNA utilized for DNA replication during PCR. Usually two primers (also called a primer pair) that flank region to be replicated by polymerase are used.

Probe
A PCR probe is a single-stranded DNA designed to bind a region of interest within the amplified fragment. During the amplification, the polymerase degrades the probe and releases a fluorescent reporter attached to the probe. The emitted fluorescence can then be quantified.

Quantitative PCR (qPCR)
An approach that allows quantification of DNA products during polymerase chain reactions based on fluorescence intensity. The fluorescent signal can be produced either by a non-specific dye binding to double stranded DNA or by a specific probe. The fluorescent signal increases with the accumulation of DNA and is then quantified against the signal produced by a known amount of DNA.

Reads
A common name used for DNA sequences generated during high-throughput sequencing.

Reference database
A collection of DNA sequences individually linked to morphologically identified specimens, which are ideally stored in museum collections. Reference databases serve to taxonomically assign DNA sequences retrieved from eDNA or bulk samples.

Replicate
Repeated DNA sampling or PCR amplification in order to estimate the variability associated with the method and control the consistency of obtained results.

Sanger sequencing
A low-throughput but high-quality DNA sequencing method used for barcoding of individual specimens.

Supervised machine learning
Statistical modelling technique that use a fully labelled dataset, to train a predictive model using a machine learning algorithm. The trained model is used to predict labels of new samples based on the distribution of their features.

Taxonomic assignment
The taxonomic identification of DNA sequences based on reference databases.

Threshold
Lowest accepted value for a given quality criterion, used at many steps during the bioinformatics process to clean HTS data.

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